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16870

Comparison of Synthetic and Fermentation Chloramphenicol (Chloromycetin) in Rickettsial and Viral Infections.

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Synthetic chloromycetin, recently prepared by Crooks and his coworkers,[1] has been shown to have the same physical properties and antibacterial activities as chloromycetin produced by the mold *Streptomyces venezuelae*, N. Sp. The present report provides comparative data on the rickettsiostatic and virustatic effects of crystalline chloromycetin obtained from two sources, *i.e.*, from fermentation liquor of cultures of *Streptomyces venezuelae*, N. Sp.[2] and from chemical synthesis.[1] Both types were supplied by the Research Laboratories of Parke, Davis and Company. Earlier publications have provided information on the use of the fermentation type of drug in the treatment of rickettsial and viral infections of experimental animals[3–7] and man.[8–11]

1 Controulis, J., Rebstock, M. C., and Crooks, H. M., Jr., *J.A.C.S.*, 1949, in press.

2 Bartz, Q. R., *J. Biol. Chem.*, 1948, **172**, 445.

3 Smadel, J. E., and Jackson, E. B., *Science*, 1947, **106**, 418.

4 Ehrlich, J., Bartz, Q. R., Smith, R. M., Joslyn, D. A., and Burkholder, P. R., *Science*, 1947, **106**, 417.

5 Smith, R. M., Joslyn, D. A., Gruhzit, O. M., McLean, I. W., Jr., Penner, M. A., and Ehrlich, J., *J. Bact.*, 1948, **55**, 425.

6 Smadel, J. E., Jackson, E. B., and Cruise, A. B., *J. Immunol.*, in press.

7 Smadel, J. E., and Jackson, E. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 478.

8 Smadel, J. E., León, A. P., Ley, H. L., Jr., and Varela, G., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 12.

TABLE I.
Comparison of Effect of Two Types of Chloromycetin in Infected Embryonated Eggs.

Infecting agent	Drug dose mg/egg	Mean prolongation of life in days of embryos treated with chloromycetin	
		Fermentation	Synthetic
R. rickettsi	.125	1.65	1.78
	.25	2.91	2.91
R. akari	.125	1.89	2.06
	.25	3.67	3.48
R. mooseri	.125	1.23	1.09
	.25	2.26	2.63
R. prowazeki	.125	1.20	1.00
	.25	2.82	2.82
R. tsutsugamushi (Gilliam strain)	.125	1.02	1.07
	.25	3.20	3.09
(Seerangayee strain)	.25	2.30	2.40
Psittacosis virus (6 BC strain)	.25	1.63	1.83
	.50	3.33	3.63
(P 4 strain)	.25	1.29	1.45
	.50	3.20	2.96
Lymphogranuloma virus (J.H. strain)	.25	3.93	3.89
	.50	7.12	7.23

Methods. The methods employed in the present work were similar to those used in previous studies [3,6–8,10] and need not be discussed.

Results. Table I summarizes the data obtained in embryonated eggs treated with varying doses of the 2 types of chloromycetin one-half hour before inoculation with a given rickettsial or viral strain. In this work the causal agents of epidemic, murine and scrub typhus, spotted fever and rickettsialpox, and of psittacosis and lymphogranuloma venereum were employed. Inspection of Table I reveals that the mean prolongations of life (MPL) of the groups infected with a given agent and treated with the same amounts of fermentation or synthetic drug were essentially identical. Statistical analysis of the basic data confirmed this. In these tests the MPL represented the difference between the mean day of death of infected embryos in treated and control groups. Each group contained 20-24 living embryos at the beginning of the period for calculating the mean day of death; depending on the infectious agent, deaths within the first 3 or 4 days after inoculation were considered non-specific.

Mice infected with the Karp strain of *R. tsutsugamushi* received a single daily intraperitoneal injection of solutions containing varying amounts of the 2 types of drug. The results obtained in one such test in which treatment was begun the day following injection of rickettsiae and continued for 20 days are summarized in Table II. Both types of drug were highly effective in preventing death of the animals.

In other experiments mice were infected intraperitoneally with the 6 BC strain of psittacosis virus and treated by intraperitoneal injection or oral administration of varying doses of the 2 types of chloromycetin. The results were essentially indistinguishable from those previously obtained[7] when the fermentation

[9] Payne, E. H., Knaudt, J. A., and Palacios, S., *J. Trop. Med. and Hyg.*, 1948, **51**, 68.

[10] Smadel, J. E., Woodward, T. E., Ley, H. L., Jr., Philip, C. B., Traub, R., Lewthwaite, R., and Savoor, S. R., *Science*, 1948, **108**, 160.

[11] Pincoffs, M. C., Guy, E. G., Lister, L. M., Woodward, T. E., and Smadel, J. E., *Ann. Int. Med.*, 1948, **29**, 656.

TABLE II.

Comparison of Chemotherapeutic Effect of Two Types of Chloromycetin in Mice Infected with *R. tsutsugamushi*.

Drug treatment I. P.		Dilution of infectious inoculum		
Type	mg/day/mouse	10^{-5}	10^{-6}	10^{-7}
None	(Controls)	8/8*	4/8	4/8
Fermentation	2.5	0/8		
	1.5	0/8		
	0.75	0/8		
	0.375	2/8		
Synthetic	2.5	0/8		
	1.5	0/8		
	0.75	1/8		
	0.375	3/8		

* Numerator = No. of mice dying; denominator = No. of mice in group.

All mice in control groups receiving 10^{-2}, 10^{-3}, 10^{-4} dilutions of challenge material succumbed while one died in the 10^{-8} group. The titer of the inoculum was $10^{-6.6}$, therefore, the drug treated mice received approximately 40 MLD's intraperitoneally.

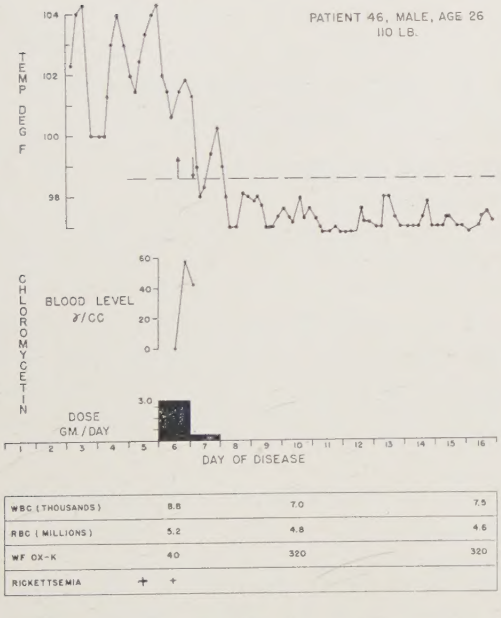

FIG. 1.

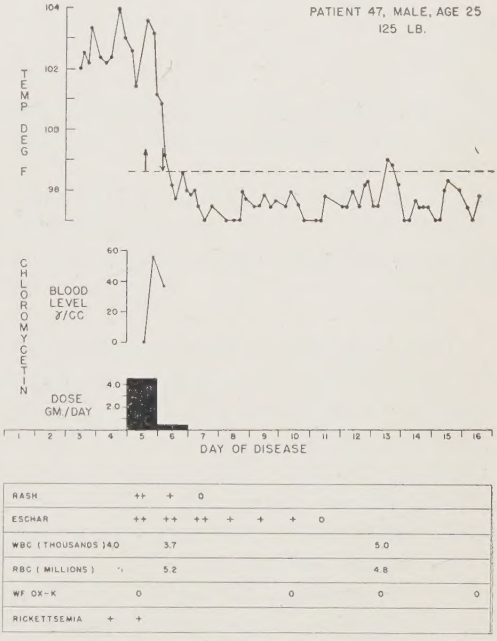

FIG. 2.

type of antibiotic was employed under similar conditions. Chloromycetin from both sources was equally virustatic in the present tests.

The clinical findings in 2 patients with scrub typhus* who were treated with synthetic chloromycetin are summarized in Fig. 1 and 2. The diagnosis was established in each individual by recovering *R. tsutsugamushi* on 2 occasions from samples of blood which were drawn during the febrile phase and promptly injected intraperitoneally into mice. Furthermore, during convalescence Patient 46 developed agglutinins for the OX-K strain of *B. proteus*. Both patients were con-

* The authors wish to thank Major C. J. Williams, RAMC, and other members of the Staff of the Military Hospital, Kuala Lumpur, who were responsible for the care of these patients.

sidered to have had typical scrub typhus although neither presented all the classical signs of the disease. Thus, Patient 46 failed to show a rash or eschar while Patient 47 failed to develop a positive Weil-Felix reaction. Both persons received the drug orally over a period of 16 hours. Patient 46 was given an initial dose of 3.0 g followed by 4 doses of 0.25 g at 3-hour intervals. The regime for Patient 47 was similar except that the initial amount was 4.0 g. The response of these 2 patients following therapy was entirely comparable to that observed in other cases of scrub typhus treated with fermentation chloromycetin. It will be recalled that the febrile period of scrub typhus, which usually lasts for 14 days in untreated cases, was terminated in 31 hours (average for 25 patients) after treatment with chloromycetin was instituted.[10]

Our experience, although limited, indicates that synthetic chloromycetin possesses the same low level of toxicity for embryonated eggs, mice and men that has characterized the fermentation type[3,5,10] of drug.

Conclusion. Chloromycetin prepared by chemical synthesis appears to possess the same rickettsiostatic and virustatic properties in experimental infections and the same usefulness in treating patients with scrub typhus that have been demonstrated for chloromycetin produced by *Streptomyces venezuelae*, N. Sp.

16871

Condensation of 2, 3-Dimercaptopropanol (BAL) with Oxophenarsine Hydrochloride: Toxicity and Chemotherapeutic Effect.

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(Introduced by E. K. Marshall, Jr.)

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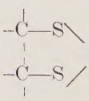
The use of 2,3-dimercaptopropanol (BAL) in the treatment of arsenical poisoning has been well established, and the pharmacological and biochemical basis for its action has been thoroughly explored. It has been shown that BAL mobilizes arsenic from tissues in which the metal has undergone a reversible binding with sulfhydryl groups in protein.[1,2] As a result BAL increases the excretion of administered arsenic from the body[3,4] and is a useful antidote in poisoning from arsenical gases or complications following administration of oxophenarsine hydrochloride.[2-7]

It has been shown that when arsenic reacts with keratin the ratio of combination with sulfur of the protein is 1 As : 2 S, and this suggested that a relatively stable ring of the type



is formed.[1] It is therefore postulated that BAL detoxifies by the removal of arsenic from the protein system and incorporation *in vivo* into a more stable cyclic

* Eli Lilly Fellow in Pharmacology and Experimental Therapeutics.

1 Stocken, L. A., and Thompson, R. H. S., *Biochem. J.*, 1946, **40**, 529.

2 *ibid.*, p. 535.

3 *ibid.*, p. 548.

4 Eagle, H., Magnuson, H. J., and Fleischman, R., *J. Clin. Invest.*, 1946, **25**, 451.

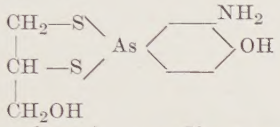
5 Longcope, W. T., Luetscher, J. A., Jr., Wintrobe, M. M., and Jagen, V., *J. Clin. Invest.*, 1946, **25**, 528.

6 Carleton, A. B., Peters, R. A., Stocken, L. A., Thompson, R. H. S., and Williams, D. I., *J. Clin. Invest.*, 1946, **25**, 497.

7 Durlacker, S. H., Bunting, H., Harrison, H. E., Ordway, N. K., and Albrink, W. S., *J. Pharmacol. and Exp. Therap.*, 1946, **87**, Supplement, 28.

thioarsenite with BAL itself.[2,8]

Three papers[9-11] have appeared dealing with some of the pharmacological properties of the thioarsenite which is formed *in vitro* by condensation of oxophenarsine hydrochloride and BAL. For convenience, the compound will be called BAL-OXO. Its structure is as follows:



However, there is a conflict concerning the toxicity of this compound relative to the parent oxophenarsine hydrochloride. Peters and Stocken,[9] using a propylene glycol solution of the hydrochloride of BAL-OXO, found that in rats it was approximately four times as toxic as the parent arsenoxide. Excess BAL reduced this toxicity markedly. On the other hand, Riker[11] found in cats that BAL-OXO was at least 4 times less toxic than the parent compound. Friedheim and Vogel[10] gave toxicity figures of BAL-OXO for the mouse and the rabbit which are considerably lower than those published elsewhere for oxophenarsine hydrochloride.[12] No direct comparison was made however. These workers also found that BAL-OXO was therapeutically active in mouse trypanosomiasis and rabbit syphilis. Although no quantitative evaluation of the drug was made, they suggested that it might be a useful chemotherapeutic agent.

The present experiments were designed to explore the relative toxicity, and therapeutic value in *T. equiperdum* in mice, of BAL-OXO and the parent oxophenarsine hydrochloride.

Materials and methods. A single strain of mice weighing 20-25 g was used throughout. Rats used for toxicity weighed 120-160 g. Animals were from Carworth Farms, Inc.

Drugs were administered in a single dose intraperitoneally. In the therapeutic experiments they were given 24 hours after a suspension of approximately 100,000 organisms of *T. equiperdum* was injected into the peritoneal cavity of the mice.

The oxophenarsine hydrochloride was a commercial preparation containing 31.8% arsenic.†

BAL-OXO was prepared as follows: To 1.88 g (.008 mols) of oxophenarsine hydrochloride in 100 ml N HCl was added 1 g (.008 mols) of 2,3-dimercaptopropanol in 20 ml methanol. Sodium hydroxide (3N) was added until neutrality was reached. A copious light pink precipitate was formed and filtered under suction. The precipitate was washed with water, methanol and ether, and dried in air. The compound was a fine light pink powder, with a melting point of 122-124°C. Analysis showed an arsenic content of 24.5% and 24.6%. The theoretical value is 24.6%. This compound was used within 3 months after it was made. There was no darkening or observable change in solubility characteristics. For solution and use in animals the following procedure was followed: 100 mg drug was dissolved in 12 ml water and 3 ml N HCl, with gentle heating. Normal NaOH was then added until the solution was only slightly acid (approx. 2.5 ml used), but no precipitate had formed. It was made up to appropriate volume and used in this form, which is the hydrochloride of BAL-OXO.

Doses of both compounds are expressed in terms of mg of arsenic per kg body weight.

In toxicity experiments all animals were observed for 14 days. Almost all deaths occurred in the first 48 hours, after oxophenarsine hydrochloride. Deaths from BAL-OXO were often delayed for 96 hours. Mice in therapeutic tests were observed for 30 days. Most deaths were in the first two weeks and none occurred beyond the twentieth day. Ten untreated infected controls died within 5 days.

Results were plotted as per cent survival against the logarithm of the dose for both toxicity and therapeutic experiments. In Fig.

8 Whittaker, V. P., *Biochem. J.*, 1947, **41**, 56.

9 Peters, R., and Stocken, L. A., *Biochem. J.*, 1947, **41**, 53.

10 Friedheim, E. A. H., and Vogel, H. J., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 418.

11 Riker, W. F., *J. Pharmacol. and Exp. Therap.*, 1946, **87**, Supplement, 66.

12 Eagle, H., Hogan, R., Doak, G. O., and Steinman, H. G., *Public Health Rep.*, 1944, **59**, 765.

† We wish to thank Dr. A. C. Bratton of Parke, Davis Company for supplying this compound in a chemically pure state.

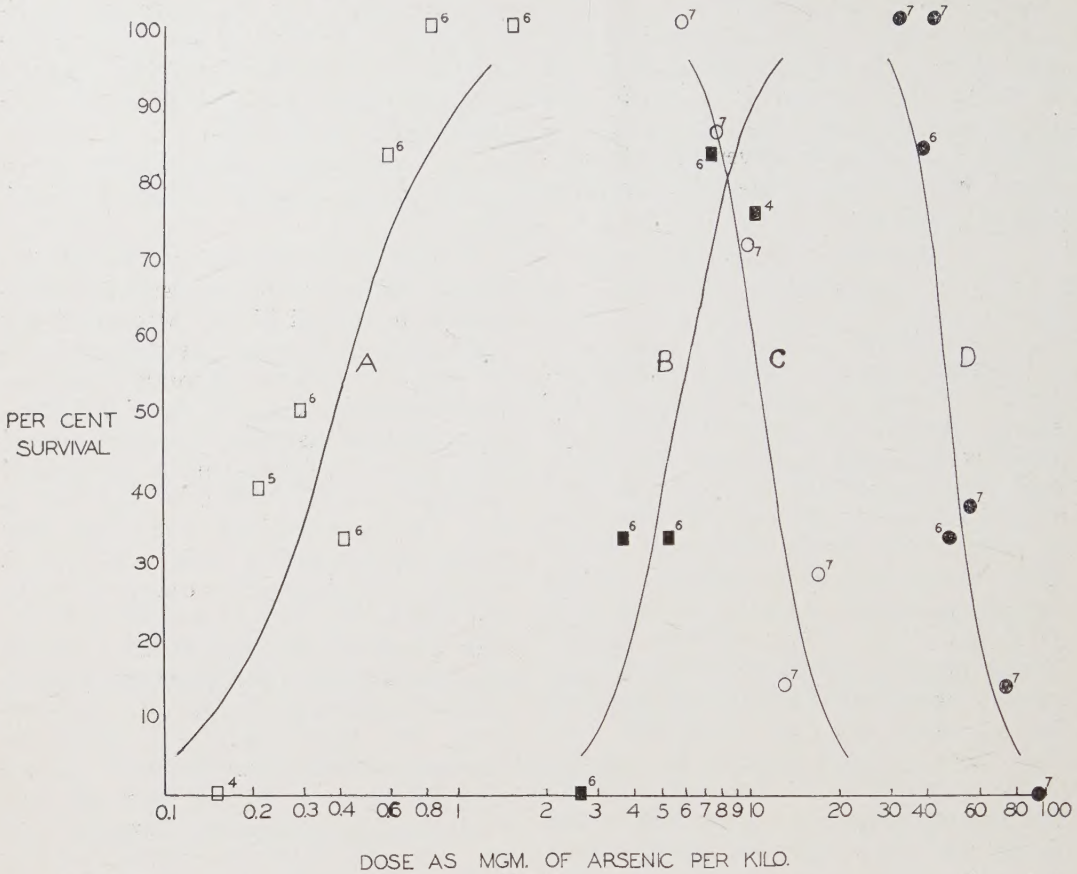


FIG. 1.

The mouse toxicity, and therapeutic effect of oxophenarsine HCl and BAL-oxo in *T. equiperdum* in mice.

Key: □ Oxophenarsine HCl therapy, curve A.
■ BAL-oxo therapy, curve B.
○ Oxophenarsine HCl toxicity, curve C.
● BAL-oxo toxicity, curve D.

The small numbers on the figure represent the number of animals used at each dose.

1, integrated normal frequency curves have been fitted to the observed data. "Chemotherapeutic index" is defined as the dose lethal to 50% of animals/dose curing 50% = LD_{50}/CD_{50}.

Results. Fig. 1 shows the data obtained for 4 experiments on mice. Smooth curves to approximate these points were constructed as described above. The acute toxicity (curves C and D) experiments were run simultaneously for the two drugs. Approximately one month later the treatment of *T. equiperdum* was run simultaneously for both drugs (curves A and B). The approximate 50% survival point may be read from Fig. 1, and will give the LD_{50} (curves C and D) and the CD_{50} (curves A and B) for both compounds. Because of the comparatively small number of animals used no attempt was made to attach a standard error to these measurements. For oxophenarsine hydrochloride the ratio LD_{50}/CD_{50} is approximately 30. This is in agreement with Eagle, Hogan, Doak and Steinman whose figure is 26.6.[12] The corresponding ratio for BAL-OXO is approximately 10.

It is evident therefore that the condensation of BAL with oxophenarsine hydrochloride yields a compound of lower "chemotherapeutic index" than the parent arsenoxide

The toxicity curves (C and D) show that

conversion of the arsenoxide to the thioarsenite has the effect of *decreasing* the acute toxicity in mice by a factor of 4. This agrees with Riker's data on cats.[11] Peters and Stocken,[9] who reported that this conversion *increased* the toxicity by approximately 4, worked entirely with rats. It was essential, therefore, to know if the disagreement was due to a species difference, and we set up several acute toxicity experiments on rats. For oxophenarsine hydrochloride, we were in fair agreement with Peters and Stocken who reported the LD_{50} as 5 mg As/kg. Our animals survived 6.5 mg As/kg and died at 10 mg As/kg. For BAL-OXO, however, there was an enormous disparity; our rats survived 32 mg As/kg and died at 64 mg As/kg, whereas Peters and Stocken reported that the LD_{50} was between 1-2 mg As/kg. It will be observed that our figures for rats agree well with our mouse data (curves C and D). Species difference alone, therefore, would clearly not account for the results of Peters and Stocken, which disagree with those of other workers, including ourselves.

Discussion. These findings suggest that in the combination of oxophenarsine hydrochloride with BAL the net toxicity for the host and for the parasite are not affected to the same degree. This agrees with Ercoli and Wilson[12] who found that relatively more BAL was needed to interfere with the toxicity of oxophenarsine hydrochloride than was needed to interfere with its trypanocidal and therapeutic effect. Both our findings and theirs, therefore, show that BAL lowers the therapeutic index of the arsenical.

The precise reason for this effect is not completely evident, but at least two hypotheses would explain the facts. First, the dithioarsenite may act as a whole and have a different relative toxicity for host and parasite than does oxophenarsine hydrochloride. On the other hand, BAL may simply alter the distribution of active arsenical between trypanosome and host. We believe that the latter view is more in keeping with all that is known of the action of arsenicals. If the dithioarsenite acts as a whole, it must act by a mechanism not explainable by the -SH-arsenoreceptor theory which is now generally accepted. For this reason, we believe that the oxophenarsine-BAL compound acts through dissociation into a compound whose arsenic is available to sulfhydryl groups in protein.

In any case, it has been confirmed that BAL-OXO does possess significant therapeutic activity, although its therapeutic index is only about 1/3 that of oxophenarsine hydrochloride. It is entirely possible that other arsenic-sulfhydryl compounds might show a greater therapeutic efficacy than the one we have studied, but from this single example it appears that combination of arsenical with a detoxifying dithiol reduces its therapeutic effect to a greater degree than its toxicity.

Regarding toxicity alone, the major discrepancy between our results and those of Riker[11] and Friedheim[10] on the one hand, and those of Peters and Stocken[9] on the other, are quite inexplicable. It is possible that differences in the preparation or stability of the drug are responsible. Secondly, they gave their compound in propylene glycol. A circumstance which suggests that Peters and Stocken were not dealing solely with the effect of the BAL-OXO is that on this drug their animals died faster than with oxophenarsine hydrochloride alone, and usually succumbed within 2 hours. In our experiments quite the reverse was noticed: even on relatively high doses, death following the thioarsenite was delayed over that of the arsenoxide.

Summary. 1. The condensation product of BAL and oxophenarsine hydrochloride (BAL-OXO) was studied. Its toxicity in mice and chemotherapeutic activity in *T. equiperdum* is reported, in comparison with similar data, for oxophenarsine hydrochloride.

2. The acute toxicity of the thioarsenite, BAL-OXO is approximately ¼ that of the arsenoxide (oxophenarsine hydrochloride) from which it was prepared.

3. The BAL-OXO has less therapeutic advantage, *i.e.,* lower LD_{50}/CD_{50}, in mouse trypanosomiasis than oxophenarsine hydrochloride itself.

16872

Requirement of the German Cockroach for Choline and Related Compounds.*

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Ordinarily choline is not considered a critical vitamin in insect nutrition, although its omission may result in retarded growth.[1] Recently, however, it was shown[2] that the omission of choline from the diet of newly hatched German roaches resulted in a complete cessation of growth within 10 days and the death of all of the nymphs within 30 days. The omission of each of the other vitamins singly resulted in less marked effects upon growth and survival.

In the present study the requirement of the roach *Blattella germanica* (L.) for choline was determined quantitatively with synthetic diets otherwise optimal for growth and maturation. Compounds related to choline were also studied. The 40-odd nymphs from a single egg sac were divided into groups of 5-7 and kept in wire-screened test-tube cages[3] at a temperature of 27-32°C. Each group received a different diet, based on variations of synthetic diet V (Table I). Food and water were given *ad libitum* and were replaced weekly, and the roaches were weighed under CO_2 anaesthesia at 10, 20, and 30 days of age. The age of maturation of each roach was also recorded.

Choline Requirement. Young roaches fed diet V (Table I) from which the choline was omitted failed to gain weight after the 10th day (Table II) and they all died before the 40th day. At a level of 500 γ of choline/g of diet V, the growth rate was very slow and only one-fifth of the insects reached maturity. At a level of 1000 γ of choline/g of diet the growth rate was improved but was still subnormal, although most of the insects matured. At both the levels of 2000 and 4000 γ choline /g of diet, growth was good, and the insects matured at an average age of 39 days, with practically all surviving. A further increase in the level of dietary choline to 8000 γ/g of diet resulted in suboptimal growth (Table II).

The finding that the cockroach requires as much as 2000 γ of choline/g of diet for optimal growth is in contrast to the experiments of Fraenkel and Blewett with other insect species[1] which were uniformly fed only 500 γ of choline/g diet. However, 2000 γ/g have been reported[4] necessary for good growth of chicks fed homocystine and a minimal level of methionine.

TABLE I.
Composition of Synthetic Diet V.

	Parts
Glucose monohydrate (*Cerelose*)	31
Casein ("vitamin-free")	30
*Cellu Flour**	30
Wesson's salt mixture†	4
Corn oil	3
Cholesterol	1
Vitamin mixture	0.6
	γ/g
Choline chloride	4000
Thiamine hydrochloride	12
Riboflavin	18
Nicotinic acid	100
Calcium pantothenate	40
Pyridoxine	16
Inositol	2000
Biotin	0.6
Folic acid	5

* Manufactured by Chicago Dietetic Supply House, Chicago, Ill.

† Wesson, L. G., *Science*, 1932, **75**, 339.

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

1 Fraenkel, G., and Blewett, M., *Biochem. J.*, 1943, **37**, 686.

2 Noland, J. L., Lilly, J. H., and Baumann, C. A., *Ann. Ent. Soc Am.*, in press, II.

3 Noland, J. L., Lilly, J. H., and Baumann, C. A., *Ann. Ent. Soc. Am.*, in press, I.

4 McKittrick, D. S., *Arch. Biochem.*, 1948, **18**, 437.

TABLE II.
Growth, Maturation, and Survival of the Cockroach *Blattella germanica* on Synthetic Diets Containing Various Amounts of Choline.

No. of times fed	Choline Cl γ/g diet	Wt of roaches 10 days, mg	20 days, mg	30 days, mg	Avg. age at matur. days	Survival at matur.
2	0	4	3	4	dead	0/11
4	500	4	5	10	80	4/22
5	1000	6	16	33	44	26/28
7	2000	6	21	44	39	34/36
6	4000	6	21	44	39	35/35
1	8000	5	19	35	45	6/6

An effort was made to demonstrate alterations in choline requirement due to changes in the level of dietary fat, cholesterol, cystine, or niacinamide. Corn oil was included at levels of 1, 3 and 10% of the diet, and cholesterol was fed at levels of 0.3, 1 and 3% of the diet. Various combinations of these 3 levels of fat and cholesterol were fed in diets containing 500, 1000 and 4000 γ of choline/g diet with no significant differences in growth rate resulting. L-Cystine and niacinamide fed at the levels of 1% and 870 γ/g, respectively, in a diet containing 1000 γ choline/g diet, likewise failed to produce significant changes in growth or maturation. It was, therefore, concluded that the requirement of the roach for 2000-4000 γ of choline/g of diet is a reasonably constant one.

Activity of Other Compounds. DL-methionine, dimethylaminoethanol, aminoethanol, or betaine hydrochloride were added to diet V in the absence of choline or as supplements to diets containing a low level of the vitamin, 500 γ/g of diet. On the choline-free diet, methionine, dimethylaminoethanol and aminoethanol had very little growth-stimulating

TABLE III.
Response of the German Cockroach to Choline and Related Compounds Fed at Equimolar Levels in Synthetic Diet V.

Exp.*	γ/g diet		Wt of roaches 10 days, mg	20 days, mg	30 days, mg	Avg. age at matur. days	Survival at matur.
	Compounds added to choline-free diet.						
C, G	Choline Cl	1000	6	12	36	44	9/11
C, D	DL-methionine†	3210	3	6	5	dead	0/10
C, D	Dimethylamino-ethanol†‡	956	4	6	7	dead	0/10
C, D	Aminoethanol	438	4	6	7	dead	0/10
C, G	Betaine HCl	1100	6	13	36	45	10/12
D	Choline Cl	2000	5	19	46	40	5/5
D	Betaine HCl	2200	5	20	48	38	11/12
G	Choline Cl	4000	6	19	38	41	7/7
G, G	Betaine HCl	4400	6	18	33	43	13/14
	Compounds added to diet containing 500 γ of choline/g.						
A, B	None		4	6	12	78	2/10
A, B	Choline Cl	1500	6	20	40	42	8/10
A, B	DL-methionine	1610	5	10	20	72	5/10
A, B	Dimethylamino-ethanol	956	6	15	24	54	8/10
A, B	Aminoethanol	657	4	6	10	92	2/10
A, B	Betaine HCl	1650	6	22	41	40	10/10

* Dietary groups included in the same series were lettered the same.

† Added at levels equivalent to choline Cl on a methyl basis. In all other experiments the compounds were added at levels equivalent to choline Cl on a molar basis.

‡ Carbide and Carbon Chem. Corp. We are indebted to R. Grunert for this preparation.

TABLE IV.
Effect of Diet Upon the Choline Content of German Cockroaches, as Determined by the *Neurospora* Method.

Insects used		Diet		Choline content	
Sex	Age, days	Basal	Composition	Range γ/g live wt	Avg
127 nymphs	3	crude	dog biscuits	900-1320	1080
2 M, 3 F	58	synth. V	choline Cl, 1000 γ/g	1520-1830	1620
3 M, 2 F	50	" III*	" Cl, 2000 γ/g	1630-1830	1730
2 M, 2 F	58	" V	" Cl, 4000 γ/g	2690-2880	2780
3 M, 3 F	58	" V	betaine HCl, 1100 γ/g	1140-1480	1290
2 M, 3 F	58	" V	" HCl, 4400 γ/g	1890-2090	2020

* Same basal diet as synth. V except vitamin K and p-aminobenzoic acid were added.

effect, since the nymphs gained only 2 or 3 mg from the 10th to the 30th days of age (Table III), and all such nymphs died before maturing. Nymphs receiving 1000 γ of choline/g diet gained 30 mg during this time. When the diets contained betaine, however, growth was equal to that of nymphs receiving an equimolar amount of choline, and the growth-promoting activity of betaine was found to equal that of choline at all the levels tested.

When the various compounds were added to a diet containing 500 γ of choline/g at levels equivalent to 1500 γ/g of choline on a molar basis, aminoethanol remained completely inactive while both methionine and dimethylaminoethanol appeared to increase growth, maturation, and survival to a considerable extent although neither compound was as active as choline itself (Table III). On the other hand the roaches fed betaine grew at least as well and matured at the same rate as those receiving choline.

Choline Content of Roaches. The successful replacement of choline by betaine in the diet of the roach in spite of the high choline requirement of this species suggested a possible interconversion of these substances *in vivo*. Accordingly, newly-hatched nymphs, and roaches which had matured on diets containing varying levels of choline or betaine were analyzed for choline with the cholineless mutant of *Neurospora crassa* 34486. The insects were starved for 48 hours, anaesthetized with chloroform and chopped with scissors into a test tube containing 10 ml of 3% H_2SO_4, and the sample was treated as described by Horowitz and Beadle,[5] except that no attempt was made to remove methionine, since the amounts of this amino acid in the roaches were too small to interfere with the determination. The mold pads were filtered from the medium, washed with distilled water, rolled into pellets and dried on a porcelain plate for 6 hours at 90°C. before weighing. The standard curve covered the range 0-20 γ choline per flask. Essentially quantitative recoveries of choline were obtained in experiments in which choline and betaine were added separately or together to aliquots of freshly chopped insects, in agreement with the finding of Horowitz and Beadle that betaine is inactive for cholineless Neurospora.[5]

The newly-hatched nymphs were found to contain an average of 1080 γ choline/g live weight (Table IV) while adults maturing on diets containing 1000, 2000 and 4000 γ choline/g diet contained an average of 1620, 1730 and 2780 γ choline/g live weight respectively. Adults which had been fed diets containing 1100 and 4400 γ of betaine hydrochloride/g of diet instead of choline contained 1290 and 2020 γ of choline /g live weight respectively. Since the newly-hatched nymphs weighed an average of 2.26 mg each, they contained only 2.4 γ choline/ insect. On the other hand the insects fed no choline but 1100 and 4400 γ of betaine/g diet weighed, on the average, 68.2 and 89.0 mg, and contained 88 and 180 γ choline/insect respectively. It is therefore evident that choline had been synthesized either by these insects or by the microorganisms associated with them. Roaches fed betaine at a molar

[5] Horowitz, N. H., and Beadle, G. W., *J. Biol. Chem.*, 1943, **150**, 325.

equivalent of 1000 γ choline/g diet contained about 80% as much choline as insects fed choline itself; at a level of betaine equivalent to 4000 γ choline/g diet, they contained 73% as much body choline as those fed choline itself. The roaches fed the higher level of betaine contained 2020 γ of choline/g live weight whereas those fed 2000 γ of choline/g of diet, the optimal amount for growth and maturation, contained only 1730 γ of choline g of live weight (Table IV). Thus, amounts of choline above those actually "needed" had accumulated in the betaine-fed group.

Discussion. The relative inactivity of aminoethanol and dimethylaminoethanol in the absence of choline suggests that in the cockroach the methylation of aminoethanol and dimethylaminoethanol by dietary methionine† must be an inefficient process if, indeed, it occurs at all. This is in contrast to the finding that aminoethanol can be methylated by methionine in the rat,[6] and that dimethylaminoethanol plus methionine supports fair growth in the chick[7] in the absence of choline.

On the diet containing a minimal level of choline the feeding of methionine, dimethylaminoethanol, aminoethanol and betaine to roaches resulted in graded growth responses which were proportional to the methyl content of the supplements, with aminoethanol being completely inactive, methionine poor, dimethylaminoethanol fair, and betaine equal to choline. Apparently, therefore some of the functions of choline can be met by methionine and dimethylaminoethanol. In this respect, the cockroach may be similar to the chick in which choline has both an "essential" and a "replaceable" role.[4] The roach is different from the chick, however, in that betaine can completely replace choline in the diet of the roach whereas it meets only the "replaceable" needs for choline by the chick.[4]

In view of the efficient synthesis of body choline by cockroaches fed betaine, and the apparent difficulty of dietary aminoethanol and dimethylaminoethanol to be methylated by methionine, it is suggested that the mechanism of choline synthesis in the cockroach may be different from that proposed[8] for the rat, *viz.*, betaine→ glycine→ ethanolamine→ choline. Rather, a direct conversion of betaine to choline is suggested.

Summary. 1. The omission of choline from an otherwise adequate synthetic diet resulted in complete failure of roaches to grow and in death shortly thereafter. For optimal growth and maturation 2000-4000 γ of choline/g of diet were found to be needed.

2. The addition of dimethylaminoethanol or aminoethanol to diets lacking choline but containing 30% of casein resulted in little or no growth. The addition of methionine, dimethylaminoethanol and betaine as supplements to a diet low in choline resulted in an increased growth response in the order named. Aminoethanol was inactive.

3. Dietary betaine replaced choline quantitatively for growth and maturation at all levels of intake, and insects fed betaine contained nearly as much choline as those fed a corresponding amount of choline.

† Synthetic diet V contains 0.9% methionine as a constituent of casein (Table I).

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16873

Relation of Fat and Protein Intake to Fatty Changes, Fibrosis and Necrosis of the Liver.*

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It is still undetermined whether the lesions seen in the livers of animals on a low protein diet or a high fat diet are all part of the same process, or are the results of two or more distinct processes. Several investigators have produced hepatic lesions of fatty change, necrosis, and fibrosis by dietary means.[1–6] Others observed the fatty change and fibrosis but no necrosis.[7–9] Daft and coworkers[4] suggested that these hepatic changes which had been considered as all having the same pathogenesis should be divided into two processes, one resulting in a cirrhosis and the other a necrosis. Himsworth and Glynn[10] reported that diets high in fat or low in lipotropic substances resulted in a fatty change followed by a diffuse fibrosis of the liver, while diets low in protein but containing lipotropic substances resulted in a massive hepatic necrosis with post necrosis scarring in animals that survived. The necrosis could be prevented by adding methionine to the diet.[11] It was also reported that necrosis could be produced by feeding diets in which the protein was supplied by an amino acid mixture omitting methionine and cystine.[12] Again methionine and also cystine prevented the necrosis. This has led to the development of the theory that dietary liver disease in rats can be divided into one type consisting of fatty change and fibrosis which is produced by a diet high in fat or carbohydrate and deficient in lipotropic substances, and a second type of damage consisting of necrosis and scarring which results from a deficiency of sulfur containing amino acids. The present study was designed to further observe the effect of a high-fat diet, and a low-protein diet, on the type of liver injury produced.

TABLE I.
Experimental Diets.

	Control diet, %	Diet I, %	Diet II, %	Diet III, %
Casein	16	16	6	4
Lard	6	51	6	6
Cornstarch	60	30	70	72
Sucrose	15	—	15	15
Salt mixture	3	3	3	3
	100	100	100	100

Methods. The animals used were male, Sprague-Dawley rats. The composition of the diets fed is listed in Table I. Each rat also

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TABLE II.
Microscopic Pathology in Livers of Animals on Diet I at Both Biopsies and at Autopsy.

Control group				Diet I		
Rat No.	Fat	Fibrosis		Rat No.	Fat	Fibrosis
1	0	0	Biopsy at 85-90 days	6	4+	0
2	0	0		7	4+	1+
3	0	0		8	4+	0
4	0	0		9	4+	0
5	0	0		10	3+	0
			Biopsy at 155-165 days	6	4+	1+
				7	4+	2+
				8	4+	1+
				9	4+	1+
				10	3+	0
1*	0	0	Autopsy at 190-200 days and	6	4+	2+
2*	0	0	animals dying earlier	7	4+	2+
3*	0	0		8	4+	2+
4	2+	0		9	4+	2+
5*	0	0		10	4+	0

* Animals dying before 190 days.

Grading of Fat

1+ Few droplets in every lobule.
2+ <half of every lobule infiltrated.
3+ >half of every lobule infiltrated.
4+ Almost all of every lobule infiltrated.

Grading of Fibrosis

1+ Strands of fibrous tissue scattered throughout section.
2+ Tissue divided into pseudolobules by fibrous bands.

received a daily oral supply of thiamin, 20 γ; riboflavin, 25 γ; pyridoxine, 20 γ; and calcium pantothenate, 100 γ. Vitamins A and D were supplied by adding 5 drops of Oleum Percomorphum§ per kilogram of diet to the melted lard as the diet was prepared.

Diet 1. The rats weighed from 140 to 170 g and one group of 13 animals were fed Diet 1, and a second group of 10 rats received the control diet (Table I). Water and food was allowed *ad libitum,* and daily food intake was measured in 5 rats of each group who were housed in individual cages. At 85-90 days liver biopsies were performed on all of the animals in the individual cages by laparotomy under ether anesthesia. The biopsy was taken from the edge of the left lobe of the liver. This procedure was repeated on the same animals in the same manner at 155-165 days. After 190-200 days on the diets the animals were sacrificed and specimens of tissue were taken from the center of the left and right lobes of the liver and from the kidney. The remaining liver of all animals was analyzed for total lipids by a modification of the method of Outhouse and Forbes.[13] All of the tissue sections were stained with hematoxylin and eosin and in addition sections stained with Sudan III were prepared from half of the animals in each group. In a few cases Masson, reticulin, acid fast stain and Best-carmine stain were also utilized.

Diet 2. Animals fed this diet weighed from 130 to 160 g. Ten rats received diet 2 and ten animals were fed the control diet (Table 1). Food was allowed *ad libitum* and daily measurement of food intake was made on five rats of each group. No biopsies were taken on these animals. Six rats in each group were sacrificed after 150 days and the remainder on the 200th day of the experiment. Tissue sections were made as outlined under Diet 1. Lipid analyses were made on half of the animals in each group.

Diet 3. The rats used weighed between 110 and 150 g. Ten animals were fed diet 3 and 10 received the control diet. All of the animals were housed in individual cages and each animal received *only* 8 g of food per day. The exact amount of food consumed by each animal was measured daily. Five

§ The authors wish to thank Dr. C. E. Bills of Mead, Johnson and Company for the Oleum Percomorphum.

[13] Outhouse, E. L., and Forbes J. C., *J. Lab. and Clin. Med.,* 1939, **25**, 1157.

TABLE III.
Microscopic Pathology of All Animals at Autopsy.

Group	Total animals	Duration of exp. (days)	No. of rats	No. died	No. with 1-2 + fat	No. with 3-4 + fat	No. with fibrosis	Total lipids mean + S.E.
Control diet	10	200		8	1	0	0	—
Diet I	13	200		7	0	13	11	20.50% ± 1.72
Control diet	10	150	6	1	2	0	0	8.98% ± 1.81
		200	4	1	0	1	0	9.40% ± 1.74
Diet II	10	150	6	1	0	6	3	28.19% ± 3.41
		200	4	1	0	4	3	23.14% ± 2.94
Control diet	10	60	5	0	1	0	0	—
		75	5	0	0	0	0	
Diet III	10	60	5	0	0	4	0	—
		75	5	0	0	4	0	—

of the animals in each group were sacrificed at 60 days and the remainder on the 75th day of the study. A single piece of tissue was taken from the left lobe of the liver for microscopic examination and all sections were stained with hematoxylin-eosin.

Results. Diet 1. The animals fed Diet 1 all showed marked fatty changes in the liver on biopsy at 85-90 days (Table II). The fat was present as large droplets which almost filled the cells, pushing the nucleus and remaining cytoplasm into a narrow rim. Fibrosis had also appeared in one animal at this time.

At autopsy of the animals fed Diet 1, almost all of the livers were enlarged and all had a yellow-brown uniform color. In many cases parts of the liver, especially the lower edge of the left and median lobes, were scarred and nodular. Microscopically the end result was a trabeculation of the entire liver by strands of fibrous tissue, dividing the liver into small pseudolobules. These strands connected central veins, portal areas, and the capsule into an interlocking network. At this stage of the lesion there was less fat histologically than seen in the biopsies, and small fat droplets were more common whereas previously large fat droplets had predominated. Some of the pseudolobules were almost fat free. The progressive nature of fibrosis was apparent when the course of each animal was followed by biopsies to autopsy.

Enmeshed in the fibrous strands were scattered, isolated, large, fat-free hepatic cells, many of which were binucleated, together with masses of a light-yellow staining substance which was found to be acid fast and assumed to be ceroid. The ceroid was rarely seen other than in the fibrous areas. The findings on all animals sacrificed at 200 days or dying before that time is summarized in Table III. Only one animal fed the control diet showed any fatty change and this was of a minor degree.

The animals fed Diet I gained weight only during the initial part of the experiment and soon reached a plateau below that of the control group (Fig. 1). During the last 4 weeks of the study respiratory infections became evident in many of the animals. Of

the 13 animals fed Diet 1, 7 died before the termination of the study on the 200th day. All had severe hepatic changes and 4 had pneumonia. Eight of the animals receiving the control diet also died of pneumonia during this period, but without hepatic pathology. The respiratory infections in the control group

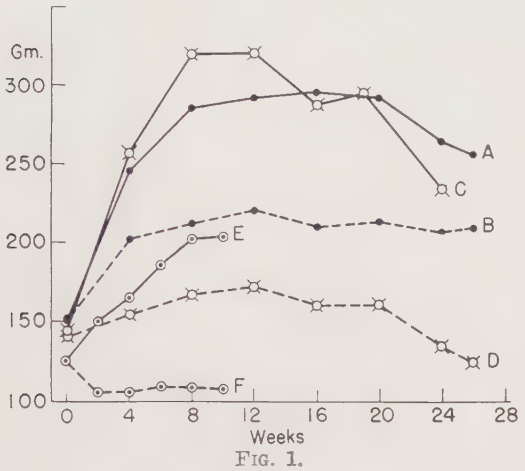

FIG. 1.

Gain in weight of rats on various diets. Curve A, control diet, and Curve B, Diet I. Curve C, control diet, and Curve D, Diet II. Curve E, control diet, and Curve F, Diet III.

is reflected in the loss of weight of this group during the last 4 weeks of observation (Fig. 1).

Diet 2. Animals fed the low protein diet (Diet 2) also showed marked fatty changes in the liver, similar to the findings in animals fed Diet 1 (Table III). Grossly the livers of the animals fed Diet 2 were enlarged and light yellow-brown in color. In some cases the edges of the left and median lobes were nodular and firm. Microscopically the type and distribution of both the fat and fibrosis in these animals were the same as those fed the 51% fat, 16% protein diet (Diet 1). No necrosis was observed either grossly or microscopically. In four of the animals the few fat-free cells that were present contained large amounts of glycogen, a finding that was not observed in animals fed the control diet. No important differences were observed histologically between the animals fed diet 2 for 150 or 200 days, except that the lesion at 200 days was slightly more advanced.

The animals fed the low protein diet (Diet 2) gained only a slight amount of weight (Fig. 1). Two of these rats died prior to sacrifice, at 125 and 164 days respectively, both with severe hepatic changes and one also had pneumonia. Two of the animals fed the control diet died from respiratory infections during this time.

Diet 3. Animals fed this diet, which contained only 4% casein, were limited to 8 g of food per day. At autopsy, either at 60 or 75 days, the rats showed hepatic changes similar to those observed with diets 1 and 2 (Table III). Grossly the livers appeared fatty, and nodularity or necrosis was not observed. Microscopically the fatty infiltration was characterized by large fat droplets, as previously described with Diet 1. There was no fibrosis or necrosis. Large deposits of glycogen, present in those cells not laden with fat, was observed in animals fed Diet 3, and not in animals fed the control diet.

The animals fed Diet 3, limited to 8 g a day, lost weight (Fig. 1). Rats receiving the control diet, also limited to 8 g of food per day, showed a moderate gain in weight.

Discussion. Although the 3 diets used were quite different the hepatic lesions produced by them appeared to be the same. Almost all of the livers were heavily infiltrated with fat. With Diet 1, a high fat diet, and Diet 2, a low fat-low protein diet, the total lipid determinations show that the actual amount of fat in the liver was the same for both diets (Table 3). Fibrosis was not seen unless fat was also present, and the fibrosis always followed the fatty change. The fibrosis was progressive, and not the type of scarring seen following an acute injury. Minor infiltrations with fat did not result in fibrosis, in agreement with other recent studies.[14] The fibrosis was not seen in the group on the 4% casein, restricted intake diet (Diet 3), as was to be expected, since all animals were killed by the 75th day, whereas animals on the other diets did not show fibrosis until after 75 days. It then appears that the fibrosis is a result of prolonged extensive hepatic fatty change which was produced by feeding a diet high in fat

[14] Glynn, L. E., Himsworth, H. P., and Lindan, O., *Brit. J. Exp. Path.*, 1948, **29**, 1.

TABLE IV.
Comparison of the Liver Injury and Protein Intake with Data of Himsworth and Glynn.

	No. of rats	Duration, days	No. with fibrosis	No. with necrosis	Food intake g/rat/day	Protein g/rat/day	Protein g/100 g/rat/day
			Diet II (6% protein, 6% fat).				
Present study	10	150-200	6	0	10.2	.614	.391
Himsworth	7	—	0	3	7.64	.458	.393
			Diet III (4% protein, 6% fat) and restricted food intake.				
Present study	10	60-75	0	0	7.6	.304	.283
Himsworth	7	—	0	4	7.18	.288	.258

or by feeding a diet low in protein.

All 3 groups of animals on the control diet contained at least one animal with some increase in liver fat. In all cases the amount of fat was small and fibrosis was absent. The slight fatty change in the control animals was associated with the presence of respiratory disease.

It is difficult to compare the findings in the present study with previous ones because of the great variation in diets and techniques. However, since the methods used here were very similar to those of Himsworth and Glynn[10] a comparison can be made for each of the diets used. In both studies the 51% fat, 16% protein diet (Diet 1) produced the same fatty changes. In the present study the appearance of fibrosis was more rapid. In both cases low protein diets (Diet 2) also resulted in fatty change but Himsworth and Glynn did not find this to be followed by fibrosis as in the present study. Some of the animals fed 6% and 4% protein diets by Himsworth and Glynn developed acute hepatic necrosis, with post necrotic scarring in the surviving animals. This lesion was not observed in our animals fed the same diets (Table IV).

Himsworth and Glynn concluded from the data collected on several low protein diets that a protein intake between 200 and 500 mg per rat per day resulted in the massive necrosis. Since our 6% protein group (Diet 2) received 614 mg the failure to develop necrosis may be explained on the basis of the higher protein intake (Table IV). The protein intake, when calculated as protein per 100 g of rat weight per day, is the same in both studies. This explanation cannot hold for the failure of the 4% protein group (Diet 3) to develop necrosis, since the animals were limited to 8 g of food per day as was done by Himsworth and Glynn, and the protein intake falls well within the critical range (Table IV). Himsworth and Glynn also found that after 40 days on such a diet many of the animals became ill and died with hepatic necrosis surviving only an average of 64 days when a 4% protein diet was fed. With a similar protein intake (Diet 3) our animals did not die during the study and failed to show hepatic necrosis, either grossly or microscopically. The average calorie and protein intake for the animals on Diets 1 to 3 is summarized in Table V.

There were some minor differences in the diets used in the two studies although the percentage of constituents was the same.

TABLE V.
Average Daily Food, Calorie and Protein Intake.

Group	Food, g per rat	Calories per rat	Calories per 100 g rat	Protein, g per rat	Protein, g per 100 g rat
Control diet	13.1	54.7	20.3	2.12	.778
Diet I	7.2	46.3	22.4	1.15	.555
Control diet	14.9	62.3	23.5	2.37	.898
Diet II	10.2	42.8	27.2	0.61	.391
Control diet	7.9	33.0	19.2	1.26	.736
Diet III	7.6	31.8	29.6	0.30	.283

Himsworth and Glynn used lard in the high fat diets and arachis oil in the low fat diets, and used cod liver oil as a source of fat soluble vitamins. In the present study lard was used in all diets and the vitamins were supplied by oleum percomorphum.

Summary. 1. Groups of rats were fed diets of 16% protein and 51% fat; 6% protein and 6% fat; and 4% protein and 6% fat. These diets resulted in a fatty infiltration of the liver, and in the long term experiments this was accompanied by a diffuse, progressive, hepatic fibrosis.

2. The hepatic lesions produced by all 3 of these diets appeared to be the same type and probably had the same basic pathogenesis.

3. Hepatic necrosis was not produced by the diets used, and deaths from acute hepatic necrosis were not obtained with a low protein intake.

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Susceptibility of the Guinea Pig to Action of Alloxan as Compared with the Rat.*

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The susceptibility to the diabetogenic as well as the general toxic action of alloxan has been studied in several species[1–9] including the human being.[10] Goldner[5] reported lesions in the islets of the guinea pig after alloxan administration but no diabetes was produced because the animals died within 24 hours. Other workers[11,12] observed changes in the blood sugar levels after alloxan injections in this species but West and Highet[13] were unable to produce alloxan diabetes in the guinea pig.

The work presented in this paper is in agreement with that of West and Highet and compares the susceptibility of the guinea pig and the rat to the action of alloxan.

Experimental. Twenty-five male guinea pigs weighing between 500 and 600 g each and kept in individual cages were divided into 5 groups. Group I consisted of 6 guinea pigs which were injected with alloxan monohydrate intravenously at various doses as shown in Table I; Group II of 7 animals partially depancreatised prior to alloxan administration. One month after the operation the animals were injected with 200 mg per kg body weight alloxan intravenously and

* Supported in part by grants-in-aid from the American Meat Institute, Chicago, Ill., the Milbank Memorial Fund, New York City, and the Nutrition Foundation, Inc., New York City.

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TABLE I.
Blood Sugar Values Following Alloxan Administration.

Treatment	Dose of Alloxan	Blood glucose in mg %: Hr after Alloxan inj. 3	8	24	48	Fasting†	Time of sacrifice after Alloxan injection
Group I	mg/kg B. wt						
24-hr fast	250	132	112	100	104		2 days
”	225	128	120	131		110	3 ”
”	225	103	88				4 ”
”	200	150		150		100	7 ”
”	200	112		150		130	13 ”
”	200	100	115	108		120	21 ”
Group II							
50% pancreatect. and 24-hr fast	200		120	112	135		2 ”
”	200	100	109	115		90	4 ”
”	200	120	118	115		130	8 ”
”	200	125	118	108		136	29 ”
”	200		125		112	108	16 ”
Group III							
24-hr fast	2080*					110	2 ”
”	2080*					105	2 ”
”	2080*					115	2 ”
”	2080*					100	2 ”
”	2080*					112	2 ”
”	2080*					118	2 ”
Group IV							
Vit. C deficiency and 24-hr fast	200	115	112			100	3 ”
”	200	125	135			113	20 ”
”	200		125	110		85	24 ”
”	200		115			90	2 ”
Group V							
3-day fasting	200		95	110	120		2 ”
”	200		100	132	130		2 ”

* Intraperitoneal injection at intervals over a 10-week period. All other groups received one intravenous injection.

† Mean value of several determinations in those cases where the time of sacrifice was later than 4 days.

were sacrificed at various intervals thereafter. Group III consisted of 6 animals which were injected repeatedly with alloxan intraperitoneally over a period of 10 weeks. A total of 2080 mg per kg was given to each guinea pig and the animals sacrificed 2 days after the last injection which was 500 mg per kg body weight. Group IV was 4 animals which were kept on a vitamin C deficient diet for a period of 36 days, prior to injection of 200 mg per kg alloxan, and Group V was 2 animals which were fasted for 3 days and then injected with 200 mg alloxan intravenously.

The intravenous injections of alloxan in all cases were made in the jugular vein after exposure of the vein with a small skin incision under ether anaesthesia. Blood samples were drawn by heart puncture and the glucose concentration was determined using the micromethod of Reinecke.[14] Urine collections were made at weekly intervals during the whole experimental period and tested for glucose using the method of Somogyi.[15]

Cytologic examination of the pancreas was made in all cases after fixing the tissue in Bouin's solution and staining by Gomori's method.[16]

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TABLE II.
Effect of Age on Response of Rats to Diabetogenic and General Toxic Action of Various Doses of Alloxan Given Intravenously.

No. of rats used	Wt, g	Dose, mg/kg	Failures, %	Mild* %	Severe† %	Deaths within 2 days %
60	80-110	50	25.0	58.3	16.7	0.0
30	90-105	60	16.7	0.0	83.3	0.0
20	87-100	70	0.0	0.0	90.0	10.0
20	85-110	80	0.0	0.0	75.0	25.0
20	280-300	40	10.0	72.5	17.5	0.0
30	280-300	50	10.0	0.0	73.3	16.7
20	290-305	60	0.0	3.0	7.0	90.0

* *Under mild* are classified those young rats which drank 40-60 cc water daily and excreted 3-5 g of sugar daily in the urine, among the adult rats those that drank 60-90 cc water and excreted 5-9 g of sugar daily.

† *Under severe* are grouped those rats that drank water and excreted glucose in the urine in amounts greater than the ones stated above.

The diet consisted of stock rabbit pellets supplemented with fresh green vegetables, carrots and 8 cc of orange juice daily, the last given by pipette. The animals in Group IV received the rabbit pellets only.

Results. Table I summarizes the data with regard to treatment, size of alloxan dose, route of administration, blood sugar levels at various intervals after alloxan, and time of sacrifice in days after the last dose of alloxan was given. The blood sugar concentration at all intervals after alloxan injection ranged from 85 to 150 mg per 100 cc of blood. These values represent post-alloxan *fasting blood sugar levels* and lie in the normal range for the guinea pig. Glycosuria was never observed.

The survival of the animals following the injections of alloxan was influenced by the size of the dose, the route of administration and the previous nutritional status. A dose of 250 or 225 mg per kg body weight killed the animals within 3 days. A dose of 200 mg was well tolerated by animals fasted for 24 hours but the same dose was fatal after a 3-day fast although the blood sugar level was in the normal range. Following the administration of 400 mg per kg by the intraperitoneal route, the animals became ill and refused to eat but had recovered by the following day.

All of the animals which received over 200 mg of alloxan per kg and those which received 200 mg per kg following a 3-day fast and which were examined during the first week after injection showed gross lesions in the lungs, liver, and kidneys. The histological examination of the pancreas showed no lesions following the intravenous administration of a 200 mg per kg dose of alloxan. The animals which received the 250 and 225 mg per kg doses had lesions of various degrees of severity ranging from cloudy swelling to necrosis and disappearance of β-cells. It was observed that doses of alloxan which were fatal produced damage in a third of the islets and only 16% of the islets were classified as severely damaged.

Discussion. The data presented indicate the resistance of the guinea pig to the diabetogenic and to the general toxic action of alloxan. The maximum tolerated dose under the conditions of these studies is approximately 200 mg per kg intravenously. Slightly higher doses cause death within a few days, the blood sugar remaining normal. For comparison, data which have been collected with rats of various sizes during the past few years are shown in Table II. It will be observed that optimum dosage for the production of diabetes in the rat apparently varies with age. Young rats tolerate 60 to 70 mg per kg of body weight in agreement with the previous studies of Mann and Stare.[17] In adult animals similar results are obtained with a dosage level between 45 and 50 mg per kg of body weight.

Several other factors besides age and dosage level are known to affect the toxity and diabetogenic action of alloxan such as the con-

[17] Mann, G. V., Stare, F. J., *J. Lab. Clin. Med.*, 1948, **33**, 1161.

centration of the alloxan solution, fasting prior to alloxan injections, and the composition of the diet.[18-21]

The development of alloxan diabetes apparently depends upon the difference in the susceptibility of the islet tissue as compared to other body tissues. If the range is sufficiently great so that marked islet destruction with only minor damage to other tissues can be obtained, diabetes can be produced. This differential is apparently found in most mammalian species. In the guinea pig, however, the islet tissue appears no more susceptible than other tissues and the resistance of both to alloxan is much greater than is found in the rat.

Summary. The parenteral administration of various dosages of alloxan, up to those which were fatal, failed to produce diabetes in guinea pigs. Neither glycosuria nor hyperglycemia was observed. Animals deficient in ascorbic acid, partially pancreatectomised or starved prior to alloxan administration, also failed to develop diabetes, although starvation apparently increased the toxicity of alloxan.

The guinea pig is much more resistant to the diabetogenic and the toxic action of alloxan than is the rat, and the pancreatic islet cells appear no more susceptible to alloxan than the cells of several other tissues.

We are indebted to Merck and Co., Inc., Rahway, N. J., Corn Industries Research Foundation, New York City, Sheffield Farms Co., Inc., New York City, and the Wilson Laboratories, Chicago, Ill., for generous supplies of materials used in these studies.

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19 Houssay, B. A., Martinez, C., *Science*, 1947, **105**, 548.

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16875

Anaphylactoid Shock Produced by Anti-Platelet Serum.*

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Decrease or even absence of platelets is well known during anaphylactic, peptonic and tryptic shocks. The rate of platelet disappearance is very rapid[1] and a relationship between the severity of anaphylactic shock and the amount of blood platelets removed from circulation has been emphasized.[2] The role of the platelet in the mechanism of these types of shock is not yet clear, but its interference has been verified.[3-5] Our purpose was to produce an anaphylactoid shock using a substance known as capable of inducing an experimental thrombocytopenic purpura. Substances known as producing experimental thrombocytopenia and purpura are estradiol benzoate[6] or urethane in the dog.[7] These substances act only after at least a week of daily administration and seem to be specific to dogs. These substances are not indicated to removing the platelets abruptly from the circulation with its sudden destruction and

* Aided by a grant from dr. Guilherme Guinle.

1 Rocha e Silva, M., and Teixeira, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 376.

2 Kopeloff, N., and Kopeloff, L. M., *J. Immunol.*, 1941, **40**, 471.

3 Achard, C., and Aynaud, M., *C. R. Soc. Biol.*, 1909, **67**, 83.

4 Rocha e Silva, M., *Rev. Brasil Med.*, 1945, **2**, 363.

5 Quick, A. J., *et al.*, *Am. J. Physiol.*, 1946, **145**, 273.

6 Arnold, O., *et al.*, *Arch. Exp. Path. and Pharmak.*, 1937, **186**, 1.

7 Cruz, W. O., and Moussatché, H., *Blood*, 1948, **3**, 793.

TABLE I.

Animals	No.	Wt (g)	Dose of normal serum* (ml/100 g body wt)	Reactions
Guinea pigs	6	270-660	1.0	None
Rats	9	170-260	0.5	"
Rabbits	5	1100-1600	0.2	"
Dogs†	4	1600-2000	1.0	‡

* Normal rabbit serum was injected into dogs, guinea pigs and rats. Normal guinea pig serum was injected into rabbits.

† Arterial pressure was taken in another dog (6.2 kg) after injection of normal rabbit serum (0.4 ml per 100 g body weight). The blood pressure after drop (4 cm Hg) came back to normal level after a few minutes and remained normal for more than ½ hour.

‡ Soon after the injections some discomfort was observed, sometimes followed by evacuation. One dog presented more acute symptoms such as vomiting and fainting. All these abnormalities disappeared within 10 minutes and the animals remained normal for hours thereafter. A typical picture of shock was not observed.

TABLE II.

Animals	No.	Wt (g)	Dose of serum (ml/100 g body wt)	Death from shock		Animals recovered showing purpura 48 hr
				No. of animals	Time, min.	
Guinea pigs	30	128-684	.30-1.0	22	2-60	8
Dogs	7	450-7200	.10-1.0	—	—	6*
Rabbits	12	1700-3200	.11- .18	3	3-15	9
Rats	7	110-240	.10- .50	5	3- 6	2

* One puppy was sacrificed 3 hours after the injection and purpuric lesions in the intestine were very conspicuous.

purpura, but anti-platelet serum is a powerful tool for this purpose and could be applied to several species. Anti-platelet serum produces a classical picture of thrombocytopenic purpura: absence or reduction of platelets in circulation, petechiae in the intestine, skin, lungs and heart; bleeding time very prolonged, normal coagulation time, clotting retraction delayed or absent and in non-fulminating cases severe anemia through intestinal hemorrhages. According to the amount administered the animal may be killed in 24 hours or, after decrease of blood platelet volume to zero, a regeneration is processed and the number of platelets becomes normal again.

We have administered intravenously a large amount of very active anti-platelet serum trying to obtain anaphylactoid phenomena in dogs, rabbits, guinea pigs and rats. The positive results of these experiments are presented in this paper.

We used 1:16 antidog-platelet, 1:16 anti-guinea pig-platelet and 1:16 antirat-platelet sera prepared in rabbits by repeated intravenous injections of platelets. The antirabbit-platelet serum was of 1:8 titre and has been prepared in guinea pigs by repeated injections in peritoneum. The platelets were obtained by fractioned centrifugation and washed several times with saline. The titration of the sera was made by the purpurigenic method.[8] 29 animals were used as control for the administration of normal serum intravenously injected in the same amounts as the higher doses in the experiments with the anti-platelet serum. The control results are shown in Table I.

Adult dogs (weight between 6.5 and 9.0 kg) have been injected with smaller doses of normal rabbit serum (one dog with 0.2 ml and 3 others with 0.4 ml per 100 g body weight). Reactions consisted in general malaise and evacuation with prompt recovery within 2 minutes after injection.

Fifty-nine animals (33 guinea pigs, 1 adult dog, 1 young dog, 5 puppies, 12 rabbits and 7 rats) have been studied. The results are summarized in Table II.

Guinea pigs. Guinea pigs were injected thru the heart. Twenty-two animals died

[8] Tocantins, L. M., *Arch. Path.*, 1936, **21**, 69.

within 1 hour (70%), 9 showed the typical signs of shock with recovery and died 24 to 48 hours later with signs of purpura. Two animals died after severe thoracic hemorrhage. Only one animal presented no symptoms of shock and died 24 hours later with signs of purpura. The picture of shock obtained was identical to those classically described: respiratory discomfort followed by violent dyspnea, urination, evacuation, convulsions and death usually within 10 minutes after the injection. At autopsy very conspicuous emphysema of the lungs was always observed. This find was confirmed by microscopic examination in several animals: edema and hemorrhage were also always present.

Dogs. The dogs were injected in the jugular or saphenous vein. With the doses employed we were not able to obtain fatal outcome. A puppy sacrificed 3 hours after the onset of shock showed a dark enlarged liver and very conspicuous purpuric lesions in the intestine. The signs of shock were agitation, urination, evacuation, vomiting, tenesmus followed by elimination of mucus with small amounts of blood, prostration and pronounced drop of body temperature.

Rabbits. The rabbits were injected in the marginal vein of the ear. Fatal shock was obtained after dyspnea and convulsions. At autopsy the characteristic extreme dilation of the right side of the heart was found.

Rats. Rats were injected in the dorsal vein of the tail. Dyspnea and convulsions were the most conspicuous signs observed. At autopsy a very typical emphysema of the lungs was found.

To observe another sign usually found in anaphylactic peptonic and tryptic shocks, *i.e.*, decrease of pressure, the carotidean pressure of some animals (2 dogs, 5 rabbits and 3 guinea pigs) was registered. One adult dog (7.2 kg) was anesthetized with liquid Dial (Ciba) the pressure drops to 50% of its normal value one minute after injection and falls as low as 5% of its initial level after 8 minutes; then the pressure rises but remains at 25% of its initial value after one hour. One young dog (3 kg) showed a strikingly similar picture. Pressure changes showed a very different picture in rabbits and guinea pigs. One rabbit anesthetized with Dial and 4 rabbits and 3 guinea pigs anesthetized with urethane showed a very uniform picture: in the first 30 to 45 seconds the pressure drops 50 to 75% from the initial level and then increases very rapidly attaining hypertensive figures from 125 to 200% of normal value, about one minute after the beginning of the injection. Those high values remain for about one minute, then drop to normal or below normal levels within a few minutes.

Numerous indications of interrelationship between signs observed in purpura and some described in anaphylactic phenomena can be pointed out. Shwartzman and Arthus phenomena have been considered allergic local hemorrhage, the mechanism of which has been long ago related to a purpuric manifestation.[9,10] Petechiae in the intestine, blood in the intestinal lumen and bloody evacuation are typical of anaphylactic shock in the dog. Thrombocytopenic experimental purpura in dogs is characterized by intestinal petechiae, blood in the intestinal lumen and severe anemia through intestinal hemorrhage, all these signs being secondary to the fall of the blood platelets.[11] Numerous cases have been described of purpura in man of an allergic nature interpreted as an anaphylactoid purpura.[12] Therapeutical substances have been imputed as causing sensitization in man producing purpuric phenomena as well as allergic reactions.[13,14]

The abrupt removal of circulating platelet by a specific serum, causing sudden destruction of this blood element accompanied by anaphylaxis-like reactions, seems also to indicate a common basic mechanism of thrombocyto-

[9] Gratia, A., and Linz, R., *Ann. Inst. Pasteur*, 1932, **50**, 89.

[10] Shwartzman, G., *et al.*, *J.A.M.A.*, 1936, **107**, 1946.

[11] Cruz, W. O., da Silva, E. M., and Pimenta de Mello, R., *Memórias Inst. Oswaldo Cruz*, 1945, **42**, 297.

[12] Glanzmann, E., *Jahrb. f. Kinderh.*, 1920, **91**, 391.

[13] Wintrobe, M. M., *Clinical Hematology*, 2nd. ed., Lea and Febiger, Philadelphia, 1947, p. 620.

[14] Watson, C. J., *et al.*, *J. Lab. and Clin. Med.*, 1947, **32**, 606.

penic purpura and anaphylactic, tryptic or peptonic shocks.

The results presented in this paper show another possible means for producing anaphylactoid shock, *i.e.*, by injecting intravenously a large amount of an active anti-platelet serum. The similarity of various signs described in purpura with some verified in allergic or anaphylactic phenomena is emphasized.

Summary. Intravenous injection of large amounts of active anti-platelet serum produces in dogs, rabbits, guinea pigs and rats a picture of severe shock quite similar to anaphylactic shock. Relationship between purpura and anaphylactic phenomena is emphasized.

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Thermolability of the Bacterium-Phage Complex.

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The infection of susceptible host cells by bacterial viruses may lead to several interesting events affecting primarily the host. A number of investigators have reported an interfering effect on the growth of unrelated viruses[1-3] while others have shown that infected cells no longer divide[2,4] or were incapable of adaptative enzyme production.[5] In the course of our investigations on the effect of sodium chloride on phage formation by staphylococci at elevated temperatures,[6] it was observed that the bacterium-phage complex was much more thermolabile than either the bacterium or phage alone. The present paper is a brief account of these observations.

The K race of phage active upon *Staphylococcus aureus* (K strain) was employed throughout these experiments. Quantitative determinations of [phage] were performed by Gratia's[7] method and viable cell counts were made by plating appropriate dilutions in tryptose agar and counting the colonies developing after 24 hours incubation at 36°C. Stock bacterial cultures were prepared by harvesting the growth from tryptose agar cultures in Roux flasks after incubation at 36°C for 18-24 hours. The suspensions were chilled for one hour in an ice-water bath and were used to make mixtures in tryptose phosphate broth containing 1 x 10^8 organisms/ml. To aliquots of these preparations phage was added in concentrations varying from 5 $\times$ 10^7 to 2 $\times$ 10^8 plaque units/ml; aliquots without added phage were maintained as controls. Samples were removed at once from the experimental mixtures and controls for determination of the viable cell counts.

As soon as samples had been taken, the tubes were immersed in the ice-water bath for an additional hour. At this time aliquots were removed for plaque determinations and viable cell counts. The latter were assumed to be a measure of the uninfected bacteria since infected cells cannot reproduce and form colonies. The plaque counts were considered to indicate the number of infected cells for it is known that phage uptake by bacteria is rapid even at 5°C and that little phage remains unattached to cells after sorption has proceeded for one hour.[8]

1 Delbrück, M., and Luria, S. E., *Arch. Biochem.*, 1942, **1**, 111.

2 Luria, S. E., and Delbrück, M., *Arch. Biochem.*, 1942, **1**, 207.

3 Delbrück, M., *J. Bact.*, 1945, **50**, 151.

4 Cohen, S. S., and Anderson, T. F., *J. Exp. Med.*, 1946, **84**, 511.

5 Monod, J., and Wollman, E., *Ann. Inst. Pasteur*, 1947, **73**, 937.

6 Fong, J., and Krueger, A. P., *J. Gen. Physiol.*, 1949, in press.

7 Gratia, A., *Ann. Inst. Pasteur*, 1936, **57**, 652.

8 Krueger, A. P., Scribner, E. J., and Brown, B. B., *J. Gen. Physiol.*, 1946, **30**, 25.

With the maximum number of cells infected by phage, the mixtures were next placed in a constant temperature bath and were held at 47.5°C for 40 minutes. In order to ascertain the effect of exposure to heat upon infected and uninfected bacteria, the plaque counts and viable cell counts were repeated.

Table I presents the data from two experiments of the sort just described. The bacterial controls exhibit no significant reduction in number of viable cells during the 40 minute period of exposure to 47.5°C, nor is there any measurable drop in the phage control. On the other hand, plaque counts in the experimental mixtures are reduced to 17% and 14% of the initial titres. The numbers of viable, uninfected cells in the experimental mixtures remain practically constant confirming the data secured from the bacterial controls.

Three other experiments besides the 2 included in Table I were performed with essentially identical results. The average value for thermal destruction of phage adsorbed to bacteria for the set of 5 experiments is 80%.

Normally, phage-infected cells are detectable as plaques; exposure of such complexes to very moderate heat treatment somehow inactivates the phage so that plaques are no longer produced. Normal, uninfected bacteria or phage alone are not affected by the experimental conditions employed. We have considered the possibility that the results secured might be due to the aggregation of the infected cells during the period of heating but no evidence of this mechanism was observable on direct microscopic examination. Furthermore, the effect of phage upon living susceptible cells is to increase the zeta potential[9] and thus to decrease the likelihood of agglutination.

Summary. Staphylococcal phage and the susceptible strain of *Staphylococcus aureus* are not destroyed during a 40 minute period of exposure to 47.5°C. However, when the phage is attached to bacterial cells the plaque count drops to approximately 20 percent of the initial value under like conditions.

TABLE I.
Effect of Heat on Bacterium-Phage Complex.
Treatment 60 min. at 4°C.

Sample	Viable $[B]_o$		$[P]_o$		Treatment	Viable $[B]_f$		$[P]_f$	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2		Exp. 1	Exp. 2	Exp. 1	Exp. 2
Bacterium, phage mixture	1.5×10^8	1.5×10^8	1.0×10^8	2.0×10^8	+ 40 min. at 47.5°C	7.8×10^7	4.5×10^7	6.7×10^7	1.0×10^8
						8.0×10^7	4.3×10^7	1.2×10^7	1.4×10^7
Bacteria control	1.5×10^8	1.5×10^8	—	—	+ 40 min. at 47.5°C	1.5×10^8	1.5×10^8	—	—
						1.4×10^8	1.4×10^8		
Phage control	—	—	1.0×10^8	2.0×10^8	+ 40 min. at 47.5°C	—	—	1.1×10^8	2.0×10^8
						—	—	1.0×10^8	2.0×10^8

$[B]_o$ Initial number bacteria/ml.
$[B]_f$ Final number bacteria/ml.
$[P]_o$ Initial number plaque units/ml.
$[P]_f$ Final number plaque units/ml.

[9] Krueger, A. P., and Mundell, J. H., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 317.

16877

Prothrombin Deficiency of the Newborn.

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(Introduced by P. György.)

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Many of the recent studies on blood coagulation have been concerned with the nature of prothrombin, its composition and the interrelationships of the various coagulation components that affect its conversion to thrombin.

Quick[1,2] at first claimed that prothrombin, as determined by the one stage method, appeared to be composed of at least two components which he designated as A and B. His original work was soon supported by other investigators[3–5] who reached similar conclusions. Seegers, Loomis, and Vanderbilt[6,7] have since prepared prothrombin that is electrophoretically homogeneous and have therefore rejected the multi-component hypothesis.

A number of recent studies, in particular those of Fantl,[8] Quick,[9] Ware,[10] Murphy,[11] Owren,[12,13] Munro[14] and MacMillan[15] are of special interest in that they present evidence of other new factors important in the conversion of prothrombin to thrombin. Ware *et al.*[16] have been able to prepare a globulin of high purity that both accelerates the prothrombin conversion and increases the yield of thrombin. Owren[13] has described a new and highly purified factor that also accelerates prothrombin conversion. It has not been settled as to whether or not this factor is identical with the substance described by Ware *et al.* MacMillan[15] has presented experiments showing that the blood of dicoumarolized patients is deficient in a factor that is different from that described by Owren.

In the present work an attempt was made to determine whether any of the previously described factors are concerned with the prolongation of prothrombin time observed in the newborn infant.

Subjects. Twelve full term infants, under 5 days of age, whose plasma exhibited a prolonged prothrombin time were selected for this study. Neither mother nor infant had received vitamin K and none showed clinical evidence of hemorrhagic phenomena. The method employed in the evaluation of the deficiency leading to the prolonged prothrombin time consisted of the addition to the infant's plasma of other plasma or serum known to contain or lack certain components. The plasma or serum added was chosen from groups of subjects as follows: 1) Plasma from 12 normal adults, (also used as controls in the prothrombin determination). Plasma from 6 normal adults stored for various lengths of time. Serum from 6 normal adults similarly stored (Series 1). 2) Plasma from 6 normal infants whose prothrombin times were within the normal range, and who had

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2 Quick, A. J., *J.A.M.A.*, 1944, **124**, 734.

3 Munro, F. L., Hart, E. R., Munro, M. P., and Walkling, A. A., *Am. J. Physiol.*, 1945, **145**, 206.

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6 Loomis, E. C., and Seegers, W. H., *Am. J. Physiol.*, 1947, **148**, 563.

7 Seegers, W. H., Loomis, E. C., and Vanderbilt, J. M., *Arch. Biochem.*, 1945, **6**, 85.

8 Fantl, P., and Nance, M., *Nature*, 1946, **158**, 708.

9 Quick, A. J., *Am. J. Physiol.*, 1947, **151**, 63.

10 Ware, A. G., Guest, M. M., and Seegers, W. H., *Science*, 1947, **106**, 41.

11 Murphy, R. C., Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1947, **151**, 338.

12 Owren, P. A., *Lancet*, 1947, **1**, 446.

13 Owren, P. A., The Coagulation of the Blood, Oslo, 1947.

14 Munro, M. P., and Munro, F. L., *Am. J. Physiol.*, 1947, **150**, 409.

15 MacMillan, R. L., *Science*, 1948, **108**, 416.

16 Ware, A. G., Guest, M. M., and Seegers, W. H., *J. Biol. Chem.*, 1947, **169**, 231.

TABLE I.—Series 1.

Prothrombin Time (Seconds) of Infants Plasmas and Mixtures with Other Sera or Plasmas as Indicated.

Infant	Infants deficient plasma (A)	Adult normal plasma (B)	75% A	25% B	Adult stored plasma (C)	Days stored	75% A	25% C	Adult serum days stored (D)	75% A 25% D
			Calculated	Observed			Calculated	Observed		
1	39.3	20.3	32	23.0*						
2	253.0	18.5	60	28.5*						
3	64.1	20.2	30	25.6						
4	37.1	19.7	27	21.5						
5	48.6	21.3	29	25.4						
6	25.7	18.9	22	18.4						
7	36.1	17.9	25	20.2	59.6	18	39	18.2	18	18.9
8	35.9	18.8	26	21.4	96.5	21	42	22.6		
9	30.7	18.8	25	21.2	83.6	50	36	18.1	50	17.4
									21	18.2
10	41.1	18.6	26	22.3	93.0	48	46	22.1	16	19.7
11	42.3	22.3	28	19.5	176.0	160	42	24.7	120	24.3
12	30.4	19.9	24.5	20.5	200.	190	30	22.3	160	27.1

* 90% A — 10% B.

TABLE II.

Prothrombin Time (Seconds) of Infants Plasmas and Mixtures with Other Plasmas as Indicated.

	Series 2				Series 3					
Infant	Infants deficient plasma (A)	Infants normal plasma (E)	75% A	25% E	Adult Dicumarolized plasma (F)	75% A	25% F	Adult Liver damage plasma (G)	75% A	25% G
			Calculated	Observed		Calculated	Observed		Calculated	Observed
4	37.1				30.9	36	32.4			
5	48.6				53.0	48	45.7			
7	36.1	19.5	26	24.1						
8	35.9	19.0	26	23.6						
9	30.7	19.7	24	23.8	34.8	27	25.8			
10	41.1	19.6	27	24.2						
11	42.3	18.9	27	25.5	33.5	39	25.5	27.5	36	30.5
12	30.4	18.2	24	24.2	31.5	30	29.9	31.5	30	32.5

not received vitamin K (Series 2). 3) Plasma from 5 adults who had received dicoumarol for thrombotic disease but were otherwise healthy (Series 3). 4) Plasma from two adults who were suffering from liver disease (Series 3).

Technic. The prothrombin times of the various plasmas and plasma mixtures studied were determined by use of the Quick method. Details are given in a previous paper.[17] The values given represent the averages (in seconds) of at least 3 determinations. Stored sera and plasmas were kept in a refrigerator at approximately 5°C for 18 to 190 days, as indicated in Table I.

Results. It will be noted that when the plasma of infants with prolonged prothrombin times was mixed with the plasma from normal adults in the proportions recorded (Table I, Series 1) a considerable shortening of the infant's prothrombin time occurred in each case, even though only a relatively small amount of adult normal plasma had been added. When the infant's plasma was mixed with adult stored plasma or serum a similar and in some cases, a more marked decrease in prothrombin time occurred. Plasma of infants with normal prothrombin times (Table II, Series 2) also decreased the prothrombin time of the infants with abnormal plasma but not as markedly as did the adult plasma. Finally when the plasma with prolonged time was mixed with plasma of dicoumarolized adults or of those with liver disease (Table II, Series 3) a variable result was obtained, but usually the decrease in prothrombin time was slight.

Discussion. Restoration of prothrombin times in the foregoing series of experiments demonstrates that more than a deficiency of prothrombin is involved, at least insofar as this is indicated by the one stage method. Considering the mixture described in the first experiment (Table I, infant No. 1), through the use of plasma dilution curves, the total theoretical prothrombin content of the mixtures may be calculated by adding the prothrombin contributed by each of the 2 component plasmas. Because of dilution, the adult plasma (assumed to be 100% before dilution) contributed 10%. The infant (containing only 20% before dilution) could contribute no more than 18%. The final mixture would be expected to contain the sum of the contributions by each plasma, or 28%, and the expected time would be 32 seconds instead of the observed 23. This discrepancy is even more obvious in the second infant studied (Table I, No. 2). This infant's plasma, having an extremely prolonged time, could contribute practically no prothrombin, and the calculated clotting time of the mixture would be that of a 10% dilution of adult plasma or 60 seconds instead of the observed 28.5 seconds. Similar reasoning applied to the other subjects is given in the column (Table I) marked "calculated". That more than a simple deficiency of prothrombin exists is further substantiated by the demonstration that the plasma of infants, normal by the one stage test, proved to be less effective than adult plasma in restoring the prothrombin times of the deficient infants.

The evidence here presented does not support the conclusions reached by Quick,[9] according to which the plasma of these infants should be deficient in the component he designates as prothrombin A. If this were so, the plasma of patients receiving dicumarol or suffering from liver disease should restore to normal the prothrombin time of the plasma of these infants. This was not evident in the seven instances studied.

Further, deficiency of Quick's[1,9] labile factor does not seem to be the basis of the deficiency seen in the newborn, because stored plasma, in which this factor is either absent or greatly diminished, proved to be as effective as fresh plasma for restoration of the prothrombin times.

Finally, and perhaps most significant was the demonstration that stored serum, from which prothrombin, thrombin and fibrinogen would have disappeared, was also effective in bringing the prothrombin times of deficient infants to normal.

It would appear from the foregoing that the prolonged prothrombin times frequently observed in the newborn, when measured by the one stage technic, cannot be adequately

[17] Randall, A., IV, and Randall, J. P., *Science*, 1948, **107**, 399.

explained as a deficiency of prothrombin itself or of any of the classical clotting factors. Rather it would seem that the newborn infant is unable to convert prothrombin to thrombin with the same speed and efficiency as the normal adult. This view would also explain the discrepancy observed in studies on such subjects in the past when their blood has been examined simultaneously by the one stage and two stage technics. Our experiments indicate that a factor is provided by normal plasma, aged plasma or serum that considerably enhances the conversion of prothrombin. Recently reported studies, as previously mentioned, have shown that one or more accelerator factors are involved in the normal blood clotting mechanism. At least one of these factors has been shown to be present in serum. It is our belief that deficiency of such a factor contributes to the clotting abnormalities of the newborn, heretofore attributed solely to a lack of prothrombin. Further investigation of this basic problem to confirm and amplify these findings is clearly required.

Two recent papers[15,18] demonstrate that a diminution of such a factor accounts for certain clotting changes observed in dicoumarolized subjects. The observation that dicoumarolized plasma exerts little effect upon the plasma of deficient infants suggests that the changes in both plasmas may be of similar nature.

Summary. 1. Evidence is presented indicating that the prolonged prothrombin times observed in the plasmas of newborn infants, studied by the one stage technic, cannot be adequately explained as a simple deficiency of prothrombin.

2. Although there may be some diminution of prothrombin, it appears that the plasma of the newborn is deficient in a factor accelerating conversion of prothrombin to thrombin. The deficiency of this factor is remedied by the addition of small amounts of normal plasma, stored plasma or serum.

[18] Owen, C. A., and Bollman, J. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 231.

16878

Agglutination of Sheep's Erythrocytes Sensitized with Histoplasmin.

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The need of sensitive and specific serologic tests in fungus diseases has lately arisen in cases with skin reactions to histoplasmin. Several publications[1–3] describing complement fixation tests have appeared. Recently Saslaw and Campbell[4] have reported agglutination tests using collodion particles sensitized with histoplasmin. The preparation of collodion particles, however, is very difficult. Middlebrook and Dubos[5] have reported a similar agglutination test for tuberculosis in which sheep's erythrocytes were sensitized with a specific substance extracted from tubercle bacilli.

The present report describes an agglutination test using histoplasmin-sensitized sheep's erythrocytes as antigen and sera from rabbits immunized with *Histoplasma capsulatum.*

* Rockefeller Foundation Fellow from University of Lund, Lund, Sweden.

[1] Tennenberg, D. J., and Howell, A., *Pub. Health Rep.*, 1948, **63**, 163.

[2] Salvin, S. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 342.

[3] Saslaw, S., and Campbell, C. C., *J. Lab. and Clin. Med.*, 1948, **33**, 811.

[4] Saslaw, S., and Campbell, C. C., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 559.

[5] Middlebrook, G., and Dubos, R. J., *J. Exp. Med.*, 1948, **88**, 521.

TABLE I.
Determination of the Optimal Dilution of Histoplasmin (Lot H-40) for Hemagglutination. Serum Prepared in Rabbits Against the G-5 Strain of *H. capsulatum*.

Dil. of histoplasmin before mixture with red cells	Immune serum dilutions										
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240
1:1	—	—	—	—	—	—	—	—	—	—	—
1:2*	+	+	+	+	+	+	+	+	±	±	—
1:3	+	+	+	+	+	+	+	±	±	±	—
1:4	+	+	+	+	+	+	+	±	±	—	—
1:6	+	+	+	+	+	+	+	±	—	—	—
1:8	+	+	+	+	+	+	±	—	—	—	—
1:12	±	+	+	+	+	+	±	—	—	—	—

Dil. of histoplasmin before mixture with red cells	Controls. Immune serum 1:10	Normal serum 1:10	Normal serum 1:20	Normal serum 1:40	Saline
1:1		—	—	—	—
1:2		±	±	—	—
1:3		±	—	—	—
1:4		±	—	—	—
1:6		±	—	—	—
1:8		—	—	—	—
1:12		—	—	—	—
Normal red cells	—				

* Optimal dilution.

Methods. Histoplasmin lot H-40† was used in this study. The red cell suspension was prepared according to the method of Middlebrook and Dubos.[5] A 0.25% suspension, however, was found to give a more sensitive test and was used instead of the 0.5% suggested in the original description.

The sera from rabbits‡ immunized with 2 strains of *Histoplasma capsulatum* (G-5 and G-6, Army Medical Center, Washington, D. C.) were used. As controls, sera from 6 normal rabbits and from 2 rabbits infected with *Sporotrichum Schenckii* (Duke strain 2079) and finally the serum from a rabbit immunized with *Candida albicans* (Duke strain) were used.

The sensitized cells-serum mixture was incubated according to the method of Middlebrook and Dubos.[5]

† Made available through the kindness of Dr. Arden Howell, Jr., Field Studies Branch, Division of Tuberculosis, Public Health Service, Federal Security Agency.

‡ Generously supplied by Miss Charlotte C. Campbell, Department of Bacteriology, Army Medical Department Research and Graduate School, Army Medical Center, Washington, D.C.

The tests were read by the pattern formed by the sedimented red cells rather than by the size of the agglutinated particles, as this was found to give more accurate readings. A positive reaction (+), as described by Salk[6] in hemagglutination tests for influenza virus, showed a thin, brownish-yellow, adherent film covering the hemispherical bottom of the test tube. In a negative reaction (—), the cells rolled to the bottom and settled as a sharply demarcated red disc which was much less adherent to the wall of the tube and moved when the tube was tilted. Reactions intermediate between the homogeneous positive film and the negative disc were characteristically ring-like in form and somewhat larger than the disc in negative tubes (±). Since reactions of this degree appeared of doubtful significance they have been regarded tentatively as negative. Serum titer was defined as the highest dilution yielding a positive (+) reaction.

Results. The optimal dilution of histoplasmin lot H-40 was found to be a 1:2 dilution (Table I).

Undiluted histoplasmin hemolysed the

[6] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

TABLE II.
Results of Hemagglutination Tests with Histoplasmin (Lot H-40).

Sera of rabbits immunized with:	Serum dilution										
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240
H. capsulatum, G-5	+	+	+	+	+	+	+	+	±	±	—
" " G-6	+	+	+	+	+	+	±	—	—	—	—
C. albicans, M-1*	±	±	±	—	—	—	—	—	—		
S. schenckii, S-7†	±	±	±	—	—	—	—	—	—		
" " S-8†	±	±	±	—	—	—	—	—	—		
6 normal rabbits	—	—	—	—	—	—	—	—	—		

* Agglutination titer with *C. albicans* cells 1:2560.
† Agglutination titer with *S. schenckii* cells 1:2560.

sheep's red cells and therefore could not be used.

As shown in Table II the sera from 2 rabbits immunized with *H. capsulatum* gave titers of 1:1280 and 1:320, respectively. Sera from normal rabbits gave negative results. Sera from 2 rabbits infected with *Sporotrichum Schenckii* and from one rabbit immunized with *Candida albicans* yielded no positive reactions. These sera were shown, however, to contain antibodies against their homologous organisms by standard agglutination tests. Serum from one human case of histoplasmosis showed no antibodies.§

It would seem, therefore, from these preliminary results that this method might be useful in serologic tests for histoplasmosis.

Summary. An hemagglutination test is described using sheep's erythrocytes sensitized with histoplasmin and sera from rabbits immunized with *H. capsulatum*. Sera from normal rabbits gave negative results and no cross-reactions were found with sera containing antibodies against *S. Schenckii* and *C. albicans*.

§ Repeated complement fixation tests of this serum, done by Miss Charlotte C. Campbell, have been similarly negative.

16879

Histological Manifestations of a Magnesium Deficiency in the Rat and Rabbit.*

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According to Tufts and Greenberg[1] two phases are observed in magnesium deficiency in rats. The first is marked by vasodilatation, hyperemia, and hyperexcitability. The second phase is characterized by the development of nutritive failure, cachexia, and kidney damage. Greenberg, Lucia and Tufts[2] showed that prolonged deprivation of magnesium eventually produces degeneration in the kidneys, histologically manifested by degenerative changes in the tubules and progressive calcification in the cortico-medullary zone, and later, in the cortex. Renal damage was also observed in calves maintained on a diet

* This work was supported in part by a grant from the Dow Chemical Company, Freeport, Texas, through the Texas A & M Research Foundation.

1 Tufts, E. V., and Greenberg, D. M., *J. Biol. Chem.*, 1938, **122**, 693.

2 Greenberg, D. M., Lucia, S. P., and Tufts, E. V., *Am. J. Physiol.*, 1938, **121**, 424.

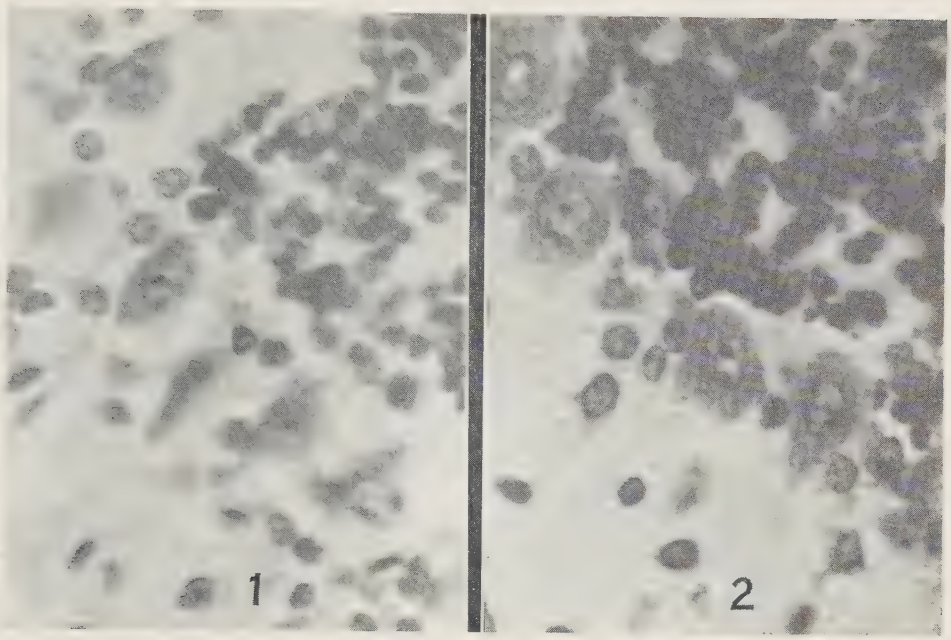

FIG. 1. Cells of Purkinje showing chromatolysis, swelling and displacement of the nucleus in a magnesium-deficient rat.

FIG. 2. Cells of Purkinje from a normal rat. (Einarson's gallocyanin chrome alum stain used in 1 and 2, × 680.)

deficient in magnesium.[3] The most consistent observation was marked proliferation of fibroblasts and fibrosis of the interstitial tissue with marked atrophy and necrosis of the parenchyma. Pathological changes in the heart due to a magnesium deficiency were reported in rats[4] and calves.[3] As Greenberg[5] has pointed out, a lack of certain B-complex vitamins has complicated the interpretations of the manifestations of a magnesium deficiency.

Bird[6] reported cerebellar lesions occurring in young chicks on a diet deficient in magnesium which were observable grossly in the severely affected chicks and only microscopically in mild cases. As far as known, no such lesions have been reported in mammals.

The work reported in this paper was designed to investigate the histological manifestations of magnesium deprivation in the cerebellum of rats and rabbits and in the kidneys and hearts of rabbits.

Experimental. Material. Cerebellar tissue was obtained from young rats that had been restricted to diets containing levels of 1 to 30 mg of magnesium per 100 g of diet for a period of 12 days. Also, cerebellar tissue was obtained from sexually mature rats that had been maintained on magnesium levels of 1 and 30 mg of magnesium per 100 g of diet for periods of 7 and 13 weeks. The dietary regimens and management of the animals has been previously reported.[7,9] In addition, cerebellar, heart, and kidney tissues were obtained from rabbits that had been started on a purified diet at weaning age and maintained on the diet which contained levels of 5 to 85 mg of magnesium per 100 g of diet for a period of 10 weeks.[8]

The brain tissue was fixed in Bouin's solution, absolute alcohol, and a solution of abso-

[3] Moore, L. A., Hallman, E. T., and Sholl, L. B., *Arch. Path.*, 1938, **26**, 820.

[4] Greenberg, D. M., Anderson, C. E., and Tufts. E. V., *J. Biol. Chem.*, 1936, **114**, xliii.

[5] Greenberg, D. M., *Ann. Rev. Biochem.*, 1939, **8**, 269.

[6] Bird, H. F., *Poultry Sci.*, 1946, **25**, 396.

[7] Kunkel, H. O., and Pearson, P. B., *Arch. Biochem.*, 1948, **18**, 461.

[8] Kunkel, H. O., and Pearson, P. B., *J. Nutrition*, 1948, **36**, 657.

[9] Einarson, L., *Am. J. Path.*, 1932, **8**, 295.

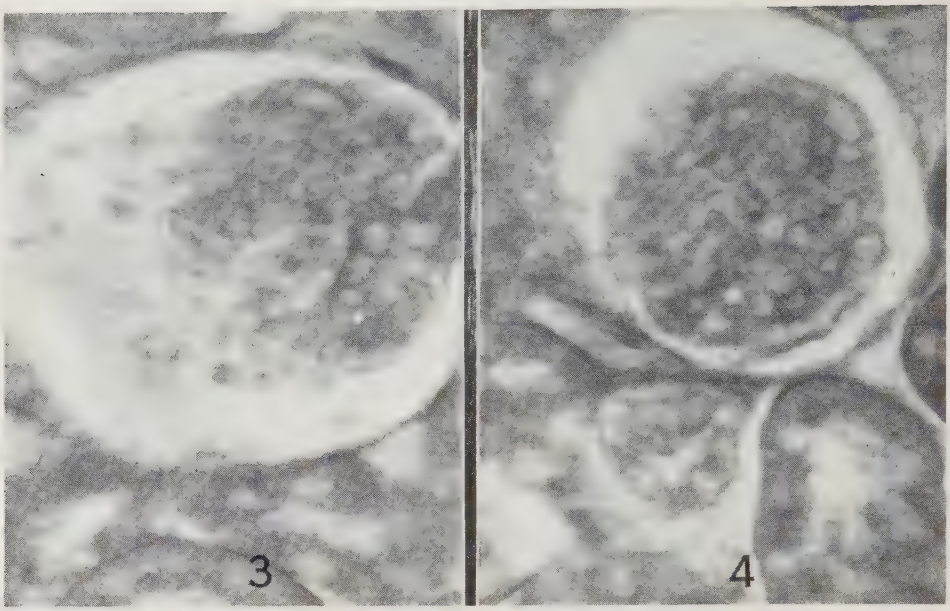

FIG. 3. Swelling and deposition of amorphous substance in the renal corpuscle of a magnesium-deficient rabbit.

FIG. 4. Normal renal corpuscle of rabbit receiving adequate level of magnesium in the diet. (Masson's trichrome stain 1 used in 3 and 4, × 680.)

lute methyl alcohol and 5% glacial acetic acid. Kidney and heart tissue were fixed in Bouin's solution and absolute alcohol. The material was imbedded in a mixture of paraffin and tissue-mat and sectioned at 7 microns. Various staining technics were used, employing silver technics, toluidine blue and erythrosin, hematoxylin and eosin, and Einarson's chrome alum gallocyanin technic.[9]

Histological observations. Histological examination of the cerebellar tissue revealed neuropathological alterations in young rats restricted to diets containing 1 mg and 5 mg of magnesium per 100 g of diet, and also in sexually mature rats restricted to a diet containing 1 mg of magnesium per 100 g of diet. At higher levels of magnesium intake, no cerebellar changes were observed.

The neuropathological changes in the rat were characterized by a degeneration of the cells of Purkinje of the cerebellum. Cytological examination revealed cells in varying degrees of chromatolysis. These cells are characterized, first, by a slight swelling, an increase in volume and a decrease in the staining property of the Nissl substance. In the second stage, there was a peripheral migration of the nucleus and an increase in vacuolation and the cell volume. The nucleus appeared to become more dense with an increase in the affinity for the stain. In some sections a number of shrunken chromatophilic cells could be observed which appeared to be the advanced stage in the degeneration of the cells of Purkinje. These observations on the neuropathological changes afford an explanation of the findings of Greenberg and Tufts[10] that protection against convulsive attacks in the magnesium-deficient rat is not afforded by the injection of magnesium salts. Also, it provides further evidence that the hyperirritability characteristic of a magnesium deficiency is not merely the result of an ion imbalance in body fluids.

Cerebellar sections from rabbits that had been restricted to diets containing 5 and 10 mg of magnesium per 100 g of diet for 10 weeks showed alteration of the cells of Purkinje. These changes were characterized by the presence of a large number of fusiform, dense and basophilic staining cells of Purkinje

[10] Greenberg, D. M., and Tufts, E. V., *Am. J. Physiol.*, 1938, **121**, 416.

without a visible nucleus. Cells with the nucleus displaced to the periphery and varying degrees of chromatolysis were frequent. The swelling that was characteristic of the degenerative changes in the rat was seldom noted. The changes in the rabbit probably represent a more advanced stage of degeneration than the picture presented by the cerebellum of the magnesium deficient rat.

Renal damage could be ascertained in sections from kidneys of rabbits maintained on a diet containing 5 mg of magnesium per 100 g of diet. This damage which involved both the renal corpuscle and tubules was not uniform throughout the kidney but was limited to certain areas of the tissue. Changes were observed in both the cortex and medulla. These degenerative changes consisted of a degeneration of the tubular epithelium and fibrosis of the corticomedullary region. Also in the renal corpuscles the capsule of Bowman remained intact as far as could be observed while the glomeruli were often displaced to the periphery by an amorphous acidophilic staining mass of material (Figures 3 and 4). Such renal corpuscles were often enlarged to as much as twice the diameter of those of the control.

X-ray examination. Sections of 2-4 mμ in thickness were removed and fixed in absolute alcohol. These sections were examined by means of an x-ray machine and plates commonly used in dental examination. Such procedure should reveal the presence of zonary calcification. No zonary calcification was observed by this technic.

Summary. Low levels of magnesium in the diet of rabbits and rats produced chromatolysis and degeneration of the cells of Purkinje of the cerebellum in the rat and rabbit. Nephrosis and fibrosis of the kidney occurs in rabbits restricted to a diet deficient in magnesium.

16880 P

Blood Oxygenation. I. The Kolff Apparatus. II. Multiple Horizontal Rotating Cylinders.*

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I. *The Kolff Apparatus.* In the course of construction of a perfusion apparatus capable of maintaining the circulation of an entire animal, an attempt has been made to use a method of oxygenation without an open blood-oxygen interface.

Experimental. Cellulose sausage casing was wound spirally around a horizontal revolving drum 50 cm in diameter and 50 cm long in a fashion similar to that of Kolff.[1] Casing of the following sizes was utilized: diam. 1.84 cm with 0.203 mm wall thickness, diam. 2.40 cm with 0.203 mm wall thickness, and diam. 4.16 cm with 0.406 mm wall thickness. The diffusion area was approximately 10,000 cm^2. An atmosphere of 95% O_2 and 5% CO_2 was maintained around the casing. Whipped beef blood at 37°C was introduced from a reservoir at one end of the spiral casing and collected at the other end. While the blood flowed through the casing, the drum was revolved at speeds varying between 17 and 100 r.p.m. Blood samples before and after passage through the apparatus were analyzed for oxygen content by the method of Van Slyke.[2] In some experiments the casing dipped into Ringer-Locke solutions to keep it wet. In order to utilize the mixing qualities as well as the diffusion surfaces of the spiral casing, small bubbles of oxygen were in-

* Aided by a grant from the United States Public Health Service, and by a research grant from the Graduate School, University of Minnesota.

1 Kolff, W. J., *The Artificial Kidney*, J. H. Kok, N.V. Hampen (Holland) 1946.

2 Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

TABLE I.
Comparison of Oxygenators.

	Kolff	Kolff with O_2 inj. into blood*	Horizontal cylinder
cc O_2 introduced into blood per min.			
Avg	5.87		20
Max.	9.73	44	24.3
Avg vol. content, cc	200	360	70
cc O_2/min. per cc blood			
Avg	.0299		.314
Max.	.0487	.122	.406
Avg blood flow, cc/min.	150	550	250

* One experiment was done to determine the maximum oxygenation obtainable under existing conditions by this method.

troduced through a 25 gauge hypodermic needle into the blood stream as it entered the apparatus. In order to obtain a significant increase in oxygenation at least 0.5 liter of O_2 per minute had to be injected into the blood.

Results. It was found that a maximum of 9.73 cc of O_2/min. could be introduced into the blood. The blood content of the casing was 200 cc at a flow rate of 150 cc/min. Thus, a maximum of 0.0487 cc of O_2 was introduced into the blood per minute per cc of blood in the apparatus. Keeping the casing wet did not increase the oxygen uptake. Introducing bubbles of oxygen directly into the blood stream negated the theoretical advantage of no open blood-oxygen interface which this apparatus presented, and foaming resulted. At a blood flow of 550 cc/min., 44 cc of O_2 per minute was introduced into the blood per cc of blood in the casing, but 14 cc of blood was lost as foam per minute.

II. *Multiple Horizontal Cylinders.* Considering that foaming and hemolysis in an oxygenator are partly due to rapid relative motion between the blood and a surface, a slowly revolving cylinder was used upon which to film the blood.

Experimental. A metal cylinder 56 cm long and 17 cm in diameter was mounted on rollers nearly horizontally, the angle being varied from 5° to 20°. Inside this cylinder, another cylinder of approximately 12.5 cm diameter was laid. A variety of cylinders 12.5 cm diameter was used, made of fine wire screen or thin acetate plastic. The plastic cylinders were perforated by numerous holes of varying size or by rectangular openings, attempting to arrive at a design which would allow blood to film on their inner surfaces and still not foam. The 12.5 cm cylinder was weighted down by a pipe so that it would roll evenly. The surface area of this arrangement was approximately 7000 cm^2. It was revolved at various speeds, but 16 r.p.m. was found to be optimal. Blood introduced at the upper end of the incline filmed the inner surface of the metal cylinder, the outer and inner surfaces of the screen (or plastic), and the outer surface of the pipe. An atmosphere of 95% O_2 and 5% CO_2 was maintained in the apparatus.

Results. With this apparatus an average of 22 cc of O_2 could be introduced into the blood per minute. At 250 cc per minute flow, 70 cc of blood were present in the film. Thus, a maximum of 0.406 cc of O_2 was introduced into the blood per minute per cc of blood in the film. However, foaming occurred and 10 cc of blood was lost as foam per minute.

Summary. 1. Cellulose sausage casing presents too great a barrier to the diffusion of O_2 into blood to make the Kolff apparatus an efficient oxygenator. 2. Although the Kolff machine mixes blood and oxygen, the foaming which results is excessive for an oxygenator. 3. Horizontal revolving cylinders oxygenate blood efficiently enough to make their use in animal perfusion practicable, but foaming is excessive.

16881 P

Blood Oxygenation. III. The Vertical Revolving Cylinder. IV. The Vertical Revolving Cone.*

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III. *The Vertical Revolving Cylinder.* An apparatus of design similar to the oxygenator of Gibbon[1,2] has been studied to determine whether its efficiency could be increased.

Experimental. A plastic (Plexiglass) cylinder 38 cm long and 18 cm in diameter (2200 cm^2 area) was mounted vertically on rollers. While the cylinder revolved, a small stream of whipped beef blood was directed onto the inner surface near the top, where it formed a film, was exposed to a 95% O_2 - 5% CO_2 mixture, and was subsequently caught in a stationary cup which fitted outside of the lower edge of the cylinder. Analyses (Van Slyke[3]) for oxygen were made on blood samples before and after oxygenation.

Results. The rate of blood flow through the oxygenator changed the volume of blood in the film. At a flow of 200 cc/min. the film content was 43 cc and at 300 cc/min. the content increased to 53 cc. Varying the rate of revolution of the cylinder (100-250 r.p.m.) did not change the volume of the film. If the blood spout was lowered half the distance to the bottom of the cylinder so that half of the cylinder area was used, the film content decreased to 25 cc at 200 cc/min. and 34 cc at 300 cc/min. Thus, the upper half of the cylinder held an average of 61% of the blood in the film when the entire drum was used. Conversely, there was an average increase of 63% in the amount of blood in the film when the area of the film was doubled, keeping the diameter of the cylinder the same. There was no foaming in this apparatus.

An average of 11.53 cc and a maximum of 20.70 cc of oxygen was introduced into the blood per minute using the entire drum. Using half of the drum area an average of 9.32 cc and a maximum of 16.50 cc of oxygen was introduced per minute. Thus, an average of 81% of the total amount of oxygen intake occurred on half of the cylinder, which held 61% of the blood. Conversely, there was a 25% increase in oxygen uptake by increasing the film content 64% (utilizing the entire drum area). Expressed in terms of the amount of oxygen introduced into the blood per minute per unit volume of film, the entire cylinder introduced an average of 0.201 cc of O_2 per min. per cc of film, while the half-cylinder introduced 0.247 cc of O_2 per min. per cc of film. This represents an increase in efficiency of 23% by using half of the drum area with the same diameter. The maximum oxygen uptake observed was 0.470 cc per minute per cc film content using half-cylinder.

Varying the rate of revolution of the cylinder between 100 r.p.m. and 250 r.p.m. did not change the rate of oxygen uptake, though the film appeared to be less even at lower speeds.

IV. *The Vertical Revolving Cone.* Observations of the vertical revolving cylinder suggested that the greatest trauma to the blood occurs when it flies off the lower edge of the cylinder into the stationary cup. By using a funnel with a slope which will permit flow of blood downward to a small hole at the center, the trauma of transfer of the film blood to a non-rotating cup is minimized by virtual elimination of centrifugal factors.

Experimental. Two funnels, one 30 cm base diameter by 40 cm high, the other 20 cm base diameter by 53 cm high were re-

* Aided by a grant from the United States Public Health Service, and by a research grant from the Graduate School, University of Minnesota.

1 Gibbon, J. H., Jr., *J. Lab. and Clin. Med.*, 1939, **24**, 1192.

2 Gibbon, J. H., Jr., and Kraul, C. W., *J. Lab. and Clin. Med.*, 1941, **26**, 1803.

3 Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

TABLE I.
Comparison of Oxygenators.

	Vertical cylinder	Half vertical cylinder	30x40 funnel	20x53 funnel	Half 20x53 funnel
cc O_2 introduced into blood per min.					
Avg	11.5	9.3	3.3	4.6	3.8
Max.	20.7	16.5	6.4	7.3	4.9
Avg vol. content, cc	48	29	42	41	26
cc O_2/min per cc blood					
Avg	.201	.247	.082	.093	.145
Max.	.390	.470	.165	.189	.195
Avg blood flow, cc/min.	250	250	200	250	250

volved in a similar manner to the cylinder, and O_2 analyses made on the blood before and after oxygenation.

Results. The efficiency of the funnel as an oxygenator was considerably less than that of the cylinder, an average of 0.10 cc and a maximum of 0.189 cc O_2 per minute per cc film content being introduced into the blood. The steeper funnel was the better oxygenator. Using 50% of the funnel area increased the efficiency as an oxygenator 56%. Varying the speed of rotation from 87-200 r.p.m. made no difference in the amount of oxygen introduced into the blood.

Summary. 1. More oxygen can be introduced into blood per unit volume of film in a vertical revolving cylinder of 18 cm diam. if segments 18 cm long are used than if the segments are 38 cm long. An increase in efficiency of 23% was obtained. 2. Speed of rotation does not, within the ranges of 100 to 250 r.p.m., affect film content or oxygenation in such a vertical revolving cylinder. 3. The vertical revolving cone does not make a good oxygenator but may conceivably be used to collect blood atraumatically from vertical cylinders.

16882

Polyethylene Plastic Needle Guides for Angiostomy.*

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A method whereby access can be gained to deep-lying vessels and chambers in the unanesthetized animal has many uses in experimental biology. London and Chlaponina[1] described a cannula for this purpose, which could be sutured to any desired large vessel and whose external end could be placed in the subcutaneous tissue. This device was used for obtaining blood samples in the course of metabolic studies. Hamilton and his coworkers[2] measured pressures in the lesser circulation through needle guides. These were constructed of metal, in two parts, and were usually seated intrapericardially in 2 operations.

The almost inert plastic, polyethylene,[3] lends itself well to the construction of needle guides that possess many advantages. These guides are prepared by blowing a suitable section of polyethylene tubing (4-6 mm o.d.) into a flattened spheroid with the aid of a

* This work was supported by the Office of Naval Research under Project N6ori-44, Task Order XI.

1 London, E. S., and Chlaponina, S. J., *Z. f. d. ges. exp. Med.*, 1937-38, **102**, 127.

2 Hamilton, W. F., Woodbury, R. A., and Vogt, E., *Am. J. Physiol.*, 1939, **125**, 130.

3 Ingraham, F. D., Alexander, E., and Matson, D. D., *N.E.J. Med.*, 1947, **236**, 362.

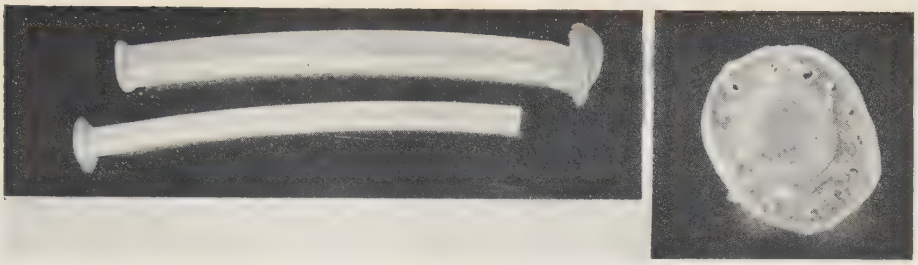


FIG. 1.
Plastic needle guide. At the left, above, is shown the guide which is sewn to the vessel or chamber. Its obturator, a segment of tubing with a cup-shaped outer end, is shown below it. At the right is an enlarged view of the foot plate showing the eyelets.

small flame. Most of the spheroid is then trimmed off leaving a curved flange which projects about 2 mm beyond the tube. A portion of the membranous segment that has been trimmed off is now faced about and sealed with a hot instrument into the flange so that its curve fits cupwise into the end of the cannula (Fig. 1). Thus the end of the tube is once again closed. This procedure is advantageous in that (1) When the needle is introduced through the guide, contact with the firm membrane at the bottom indicates that the wall of the vessel is immediately beneath. (2) After penetration, the polyethylene supports the tip of the needle and aids in keeping it from slipping too far across the lumen with possible injury of the opposite wall. (3) The membrane acts as a tampon in controlling any hemorrhage from the wall when the needle is withdrawn.

Perforations are made circumferentially through the flange in the manner illustrated in Fig. 1. These eyelets serve for suturing the guides intrapericardially to the external coats of the pulmonary artery and left auricle respectively. The attachment to the auricle is made at the posterior pericardial reflection. Sterilization of the polyethylene can be accomplished with little distortion by boiling or by soaking in an appropriate solution. Care is exercised in bringing the free ends of the cannulas to the subcutaneous position without distorting the pulmonary artery or left auricle. The cannula from the former can be brought out anteriorly along the mediastinal pleura; that from the left auricle is oriented in the interlobar fissure so as not to compress the structures of the hilum. The guides are cut to appropriate length with a scalpel. Their free ends are fixed by suture in the subcutaneous tissue of the lateral aspect of the thorax. An obturator of smaller polyethylene tubing with a cup-shaped external end may be introduced into each cannula. The chest is then closed in layers, taking care to reexpand the lungs.

The inert polyethylene in the entire operative procedure produces minimal adhesions. Healing usually is rapid and uneventful.

Blood may be obtained or pressures measured with the Hamilton manometer or other suitable means, by introducing 18-gauge "spinal" needles through the sterilized skin into the guides. If difficulty is encountered, the head of the guide can be exposed in a very short incision after anesthetizing the skin which is first displaced in a fold so made that the line of incision will later not overlie the cannula.

Summary. The construction of a polyethylene plastic angiostomy cannula is described. This cannula has many advantages for manometry of otherwise inaccessible structures in the intact animal.

16883

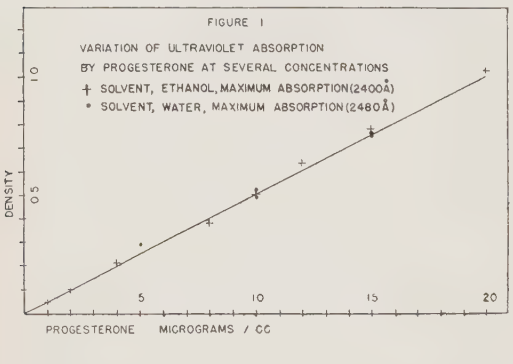
Solubility of Progesterone in Water and in Saline.*

ARTHUR L. HASKINS, JR. (Introduced by Willard M. Allen.)

From the Department of Obstetrics and Gynecology, Washington University School of Medicine, Saint Louis, Mo.

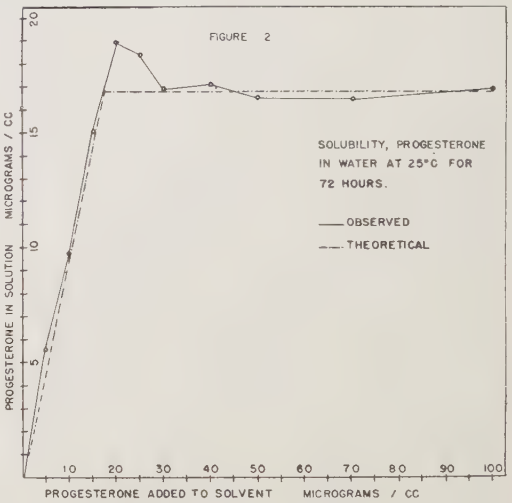
During the course of studies concerning the metabolism of progesterone, it became expedient to determine the solubility of this hormone in distilled water and in 0.9% aqueous saline. Because of the paucity of published data in this regard and its application to both *in vitro* and *in vivo* metabolic studies of progesterone, the findings are reported at this time.

The progesterone used in this study was synthetic alpha-progesterone, melting point of 128°C, and with characteristic ultraviolet absorption band at 2400 Å when dissolved in 95% ethyl alcohol. The Beckman

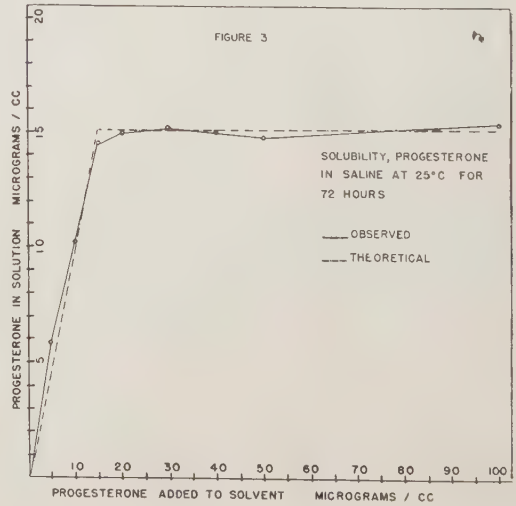


spectrophotometer, with 1 cm square fused silica absorption cells, was used in making all ultraviolet absorption observations. Fig. 1 illustrates the relation of concentration of progesterone in solution, to ultraviolet density obtained. It is apparent that progesterone whether in aqueous or alcoholic solutions follows Beer's law.

Into chemically clean 10 ml volumetric flasks, various concentrations of progesterone in 1 ml of 95% ethanol were introduced. The concentrations varied from 50 μg/ml to 1000 μg/ml. These alcoholic solutions were evapo-

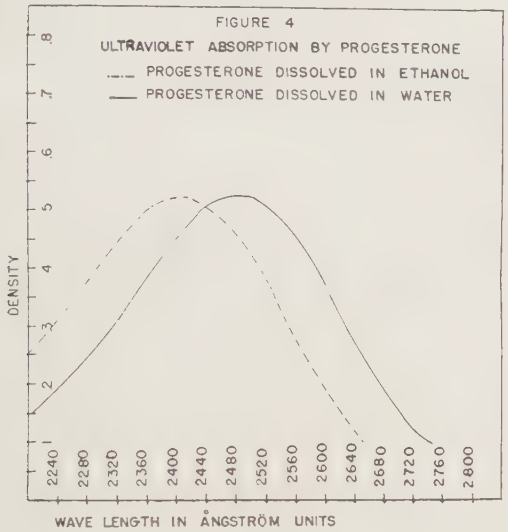


rated to dryness on a steam bath. To the residue was added 2 ml of the intended solvent, either distilled water or aqueous NaCl (0.9%). This mixture was returned to the steam bath for 30 minutes, then removed, cooled, and diluted to a 10 cc volume with the appropriate solvent. The flasks were then placed in a closed room with mean tempera-



* United States Public Health Fellow in Obstetrics and Gynecology.

ture of 25° C until spectrophotometric readings were made at 24, 48, 72 and 168 hour intervals on the supernatant fluid. The solubility of progesterone thus obtained is presented in Fig. 2 and Fig. 3. It should be noted that equilibrium between the progesterone and its solvent was obtained at 72 hours and that the solution concentration remained constant to 168 hours. The slight irregularity in solubility noted in Fig. 2 is thought to represent supersaturation.



An incidental finding noted during these solubility studies was the shift of maximum ultraviolet absorption by progesterone from 2400 Å when dissolved in 95% ethanol to 2480 Å when dissolved in water. This shift occurred quite independently of the pH of the solution. Ultraviolet absorption curves are presented in Fig. 4 to illustrate this finding.

As will be noted in Fig. 2 and Fig. 3 the average solubility of progesterone in distilled water at room temperature for 72 hours was found to be 16.8 μg per ml and in 0.9% aqueous saline, 15.1 μg per ml, under identical conditions.

The solubility of progesterone in aqueous media as found in this experiment is at variance with that reported by Forbes and Hooker[1] of 6-9 μg per ml in saline. These results should not be construed as a marked disagreement since the smaller recorded solubility was based on bioassay primarily and the limited quantitative accuracy of bioassay is admitted.

Summary. The solubility of crystalline progesterone in aqueous media was determined spectrographically. Progesterone was found to have an average solubility at room temperature of 16.8 μg per ml in distilled water and 15.1 μg per ml in 0.9% aqueous saline.

[1] Forbes, T. R., and Hooker, C. W., *Science*, 1948, **107**, 151.

16884

Cell Proliferation Accelerating and Inhibiting Substances in Normal and Cancer Blood and Urine.*

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The rate of cell proliferation of bone marrow cells and of normal tissue cells cultured *in vitro* is accelerated by normal blood serum and inhibited by blood serum from cases of neoplastic disease, pernicious anemia, aplastic anemia and leukemia.[1] These pathological blood sera and normal blood sera counteract the effect of each other in a manner similar to the counteracting effect of xanthopterin and antixanthopterin[2] on cell proliferation *in*

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

[1] Norris, E. R., and Majnarich, J. J., *Am. J. Physiol.*, 1948, **153**, 483.

[2] Norris, E. R., and Majnarich, J. J., *Am. J. Physiol.*, 1948, **152**, 652.

TABLE I.

The Effect of Fractions Obtained from Normal and Pathological Urine upon the Rate of Cell Proliferation in Bone Marrow Cultures *in vitro*. Time of incubation, 7 hours. The initial concentration of the bone marrow cell suspension was: red blood cells (RBC) 8400/cmm; nucleated cells (NC) 4760/cmm; reticulocytes 11/1000 RBC.

Supplement added	Final Concentration, RBC/cmm	RBC % Increase	Final conc., NC/cmm	NC % Increase	Retic. per 1000 RBC
No supplement	10960	28	7920	66	16
5 γ xanthopterin/ml	14300	70	10600	123	32
5 γ 7-MP*/ml	4400	—48	2960	—38	0
Normal Human Urine					
Original urine	13500	61	10500	121	30
Norit filtrate	10700	27	8440	77	30
NaOH eluate	8640	2	6520	37	12
NH_3-acetone eluate	15300	82	11800	148	36
Carcinoma of the Prostate					
Original urine	13800	64	10400	118	30
Norit filtrate	10600	26	6800	43	20
NaOH eluate	4850	—42	3040	—36	4
NH_3-acetone eluate	10600	26	8600	81	22
Broncheogenic Carcinoma					
Original urine	13500	61	9080	91	28
Norit filtrate	9700	15	6850	44	20
NaOH eluate	4680	—44	2280	—52	2
NH_3-acetone eluate	10800	29	9000	89	16
Gastric Carcinoma					
Original urine	15100	92	10100	112	30
Norit filtrate	8900	6	7320	54	16
NaOH eluate	4350	—48	2000	—58	6
NH_3-acetone eluate	11700	39	8250	74	16
Hyperrenal Carcinoma					
Original urine	13600	62	8700	83	30
Norit filtrate	9050	8	6080	28	20
NaOH eluate	4800	—43	1840	—61	2
NH_3-acetone eluate	12300	46	8750	84	16
Aplastic Anemia					
Original urine	11900	42	8680	82	22
Norit filtrate	10700	27	7000	47	14
NaOH eluate	4320	—49	2200	—54	2
NH_3-acetone eluate	10600	26	7520	81	24
Carcinoma of the Rectum					
Original urine	12880	53	10000	110	26
Norit filtrate	10440	24	8520	79	20
NaOH eluate	4520	—46	2640	—45	2
NH_3-acetone eluate	12300	46	8750	84	24

* 7-MP indicates 2-amino-4-hydroxy-7-methyl pteridine.

vitro. It was suggested[1] that all blood sera contained both stimulating and inhibiting substances in varying ratios, such that normal blood serum contained a predominance of substances which would accelerate normal cell proliferation *in vitro* and blood serum from certain diseases contained a predominance of substances which would inhibit cell proliferation of normal cell suspensions and accelerate proliferation of neoplastic tissue cells.

If both accelerating and inhibiting substances are present in biological fluids, they might be separated to demonstrate their presence. For this purpose normal and pathological blood serum and urine was fractioned by adsorption on norit and elution as decribed below.

The proteins of a given volume of blood serum were precipitated with an equal volume of acetone. After mixing and filtering the precipitated proteins, the filtrate and washings were evaporated to small volume under reduced pressure to remove the acetone. The concentrated solution was diluted with distilled water to the original volume of blood serum used. The deproteinated serum extracts and urines were treated in the same manner in adsorption and elution.

An aliquot, usually 10 ml of the blood serum extract prepared as indicated above or of urine was shaken with norit and filtered. The filtrate was saved for testing on cell proliferation.

The norit was eluted with normal NaOH and filtered. The NaOH filtrate and washings were neutralized and made up to the original volume of the blood serum or urine used. The NaOH eluate was saved for testing on cell proliferation.

After elution with NaOH, the norit was eluted with ammoniacal acetone containing equal parts of 0.4M ammonium hydroxide and acetone. The filtrate and washings were concentrated under reduced pressure to small volume to remove the acetone and ammonia. The concentrated solution was diluted to the original volume of the serum or urine used for adsorption and saved for testing on cell proliferation as the NH_3 acetone eluate.

The effect of the fractions obtained was tested on bone marrow cultures *in vitro* by the technique as previously described.[2] The fractions were made up to a volume such that aliquots could be used as supplements in the bone marrow cultures equivalent to 0.1 ml of the original serum or urine for comparison. The results are shown in Tables I, III and IV. Controls are given with no supplement, xanthopterin, 2 - amino - 4 - hydroxy-7-methyl pteridine (7-MP) and Vitamin B_{14}.[3] One x 10^{-7} γ per ml of Vitamin B_{14} accelerates the rate of cell proliferation as great or greater than 5 γ per ml of xanthopterin, or Vitamin B_{14} is at least 50 million times as effective in accelerating cell proliferation in a bone marrow culture *in vitro* as xanthopterin.

Table I gives the results for fractionation of normal and pathological urines. Although neoplastic blood serum contains a predominance of inhibiting substances, the urines from cases of neoplastic disease had a predominance of substances which accelerate the rate of cell proliferation. When 0.1 ml of urine, from the neoplastic cases tested, was added to 2 ml of bone marrow suspension, the rate of cell proliferation was similar to that obtained with 0.1 ml of normal urine. The NaOH eluate contained an excess of inhibiting substances. The NaOH eluate from normal urine produced a rate of cell proliferation less than that of the control, which contained no supplement, indicating the presence of an inhibiting substance. The NaOH eluate from pathological urines all inhibited cell proliferation strongly, and had an activity similar to that of 5 γ per ml of 2-amino-4-hydroxy-7-methyl pteridine.

Table II gives the effect of fractions from neoplastic urine and other factors on the rate of cell proliferation in a suspension of cells of Brown Pearce tumor. The tumor cells were cultured by the technique previously described.[4] The effect of the pteridines, normal blood serum, and pathological blood serum on cultures of cells of neoplastic tissue *in vitro* is opposite to the effect on suspensions of normal cells.[4] Folic acid (pteroyl glutamic acid) and teropterin (pteroyl triglutamic acid) had no effect. Xanthopterin, Vitamin B_{14} and normal blood serum inhibited cell proliferation. Two-amino-4-hydroxy-7-methyl pteridine and cancer blood serum accelerated the rate of cell proliferation. The urine of broncheogenic carcinoma which accelerated

TABLE II.

Effects of Fractions from Pathological Urine Compared with Those of Other Factors on Cell Proliferation of Cells of Brown Pearce Tumor *in vitro*. The initial concentration of cells in the suspension was 18700/cmm. Time of incubation, 6 hours.

Supplement added	Final conc. of cells	% increase in cells
No supplement	21000	12
10 γ/ml folic acid	21200	13
10 γ/ml teropterin	21600	15
5 γ/ml xanthopterin	10700	—43
10 γ/ml "	8080	—57
10^{-3} γ/ml Vit. B_{14}	10200	—45
0.1 ml normal blood serum	9950	—47
5 γ/ml 7-MP*	29000	55
10 γ/ml 7-MP*	31200	67
From Broncheogenic Carcinoma		
0.1 ml blood serum	29400	57
Original urine	13000	—30
Norit filtrate, from urine	25700	37
NaOH eluate, from urine	31000	66

* 7-MP indicates 2-amino-4-hydroxy-7-methyl pteridine.

[3] Norris, E. R., and Majnarich, J. J., *Science*, in press.

[4] Norris, E. R., and Majnarich, J. J., *Am. J. Physiol.*, 1948, **153**, 492.

TABLE III.

The Effect of Fractions Obtained from Normal Rat Serum upon the Rate of Cell Proliferation in Bone Marrow Cultures *in vitro*. The initial concentration of the bone marrow cell suspension used was: red blood cells (RBC) 6920/cmm; nucleated cells (NC) 4280/cmm; and reticulocytes 11/1000 RBC. Time of incubation, 5½ hours. All supplements were adjusted equivalent to 0.1 ml of the original serum.

Supplement added	Final Concentration, RBC/cmm	RBC % Increase	Final conc., NC/cmm	NC % Increase	Retic. per 1000 RBC
No supplement	7680	11	5360	25	18
5 γ Xanthopterin/ml	12800	85	8700	103	32
1 $\times$ 10^{-7} γ Vit. B_{14}/ml	14900	116	8400	96	34
1 $\times$ 10^{-6} γ " "	18000	160	11100	159	40
5 γ 7-MP*/ml	3820	—45	1960	—54	4
Normal Rat Blood Serum					
Original serum	14200	104	7500	75	30
Norit filtrate	8360	19	5100	19	18
NaOH eluate	4240	—39	4560	6	4
NH_3-acetone eluate	18800	173	12900	202	40

* 7-MP is used to indicate 2-amino-4-hydroxy-7-methyl pteridine.

the rate of cell proliferation of bone marrow cells *in vitro*, inhibited the proliferation of cancer cells of Brown Pearce tumor. The NaOH eluate from the cancer urine, which strongly inhibited cell proliferation in bone marrow cultures, strongly accelerated the cell proliferation in a suspension of cancer cells of Brown Pearce tumor. This showed that while in the original cancer urine there was a predominance of factors which accelerated normal cell proliferation and inhibited cancer cell proliferation a fraction was obtained as the NaOH eluate which had a predominance of substances which inhibited normal cell pro-

TABLE IV.

The Effect of Fractions Obtained from Normal and Pathological Human and Rabbit Blood Serum upon the Rate of Cell Proliferation in Bone Marrow Cultures *in vitro*. The initial concentration of the bone marrow cell suspension used was: red blood cells (RBC) 11,640/cmm; nucleated cells (NC) 4880/cmm; and reticulocytes 10/1000 RBC. All supplements added were adjusted equivalent to 0.1 ml of the original serum.

Supplement added	RBC % increase	NC % increase	Retic. per 1000 RBC
No supplement	12	24	20
5 γ xanthopterin/ml	29	77	30
5 γ 7-MP*/ml	—47	—53	4
1 $\times$ 10^{-7} γ Vitamin B_{14}/ml	60	108	40
Normal Human Blood Serum			
Original serum	26	75	36
Norit filtrate	9	30	20
NaOH eluate	—40	— 8	8
NH_3-acetone eluate	40	57	36
Serum from a Case of Broncheogenic Carcinoma			
Original serum	—35	—27	6
Norit filtrate	8	— 7	6
NaOH eluate	—79	—75	0
NH_3-acetone eluate	33	75	22
Normal Rabbit Blood Serum			
Original serum	34	81	32
Norit filtrate	7	36	18
NaOH eluate	—33	6	8
NH_3-acetone eluate	58	118	40
Serum of Rabbit Brown Pearce Tumor Blood Serum			
Original serum	—32	—28	2
Norit filtrate	1	6	10
NaOH eluate	—70	—72	0
NH_3-acetone eluate	32	81	22

* 7-MP is used to indicate 2-amino-4-hydroxy-7-methyl pteridine.

liferation and accelerated cancer cell proliferation.

Table III gives the effect of fractions obtained from normal rat serum upon the rate of cell proliferation in bone marrow cultures *in vitro*. The normal rat serum accelerates the rate of cell proliferation. The NaOH eluate inhibits proliferation and the NH_3-acetone eluate accelerates the rate of cell proliferation more strongly than the original blood serum, when used in amounts equivalent to the original blood serum. While the fractionation was not quantitative and the substances which inhibit and accelerate the cell multiplication were not isolated, it shows definitely that both types of substances were present in the original blood serum.

TABLE V.

The Effect of Fractions from Normal Blood Serum upon the Rate of Cell Proliferation in Brown Pearce Tumor Cell Suspension, *in vitro*. Time of incubation was 7 hours. The initial cell concentration of the suspension was 9600/cmm.

Supplement added	Final conc. cells/cmm	% increase of cells
No supplement	11900	24
5 γ/ml xanthopterin	6200	—35
5 γ/ml 7-MP*	17300	80
1×10^{-6} γ/ml Vitamin B_{14}	4360	—54
1×10^{-7} γ/ml Vitamin B_{14}	7160	—25
Normal Human Blood Serum		
Original serum	6320	—34
Norit filtrate	13400	50
NaOH eluate	16000	67
NH_3-acetone eluate	7240	—24
Normal Rabbit Serum		
Original serum	7920	—12
Norit filtrate	11760	23
NaOH eluate	18600	94
NH_3-acetone eluate	6760	—29
Normal Rat Serum		
Original serum	7240	—24
Norit filtrate	13180	48
NaOH eluate	18920	97
NH_3-acetone eluate	4840	—50

* 7-MP indicates 2-amino-4-hydroxy-7-methyl pteridine.

Table IV gives the effect of fractions obtained from normal and pathological human and rabbit blood serum upon the rate of cell proliferation in bone marrow cultures *in vitro*. The supplements of serum and fractions are all equivalent to 0.1 ml of the original blood serum in 2 ml of bone marrow suspension. Normal blood sera accelerated the rate of cell proliferation. Cancer blood sera inhibited cell proliferation. The NaOH eluates all inhibited proliferation and those from the cancer blood serums inhibited more strongly than the original serum. The NH_3-acetone eluates accelerated the rate of cell proliferations. While the Norit filtrates appeared to be somewhat inhibitory, nothing definite can be said about them at this time as the adsorption on Norit may not have been complete for all factors. The results show that there was present, in all urines and blood sera studied, two types of factors which influence the rate of cell proliferation, one type which accelerates the rate of proliferation and one type which inhibits cell proliferation.

Table V gives the effect of the fractions from normal human, rabbit and rat sera shown in Tables III and IV upon the rate of proliferation of Brown Pearce tumor cells *in vitro*. The technique used was the same as that previously described.[4] The original serum, Norit filtrate and eluates were so adjusted in the experiment that each was equivalent to 0.1 ml of the original blood serum in 2 ml of tumor cell suspension. The results are opposite to those obtained on bone marrow culture, which indicates that the NaOH eluate contained factors which inhibited the normal cell proliferation and accelerated the rate of cancer cell proliferation; and the NH_3-acetone eluate contained factors which accelerated the rate of normal cell proliferation and inhibited cancer cell proliferation. The two types of factors were shown to be present in each of the original sera tested, and present in such a ratio that the net result of the original serum exhibited a predominance of factors which accelerated the rate of normal cell proliferation and inhibited cancer proliferation.

Conclusions. 1. Blood serum and urine contain two types of substances; the one which accelerates the rate of cell proliferation, and the other which inhibits the rate of cell proliferation.

2. Normal blood sera and normal and pathological urine accelerate the rate of cell proliferation in bone marrow cultures *in vitro* because of an excess of accelerating substances over inhibiting substances present.

3. Blood sera from neoplastic disease, pernicious anemia and leukemia inhibit cell pro-

liferation in bone marrow cultures because of an excess of inhibiting substances over accelerating substances present.

4. Individual urine and blood serum specimens have been shown by adsorption on Norit and elution with NaOH and ammoniacal acetone to have both inhibiting and accelerating substances present.

16885

The Effect of Methionine on Blood Coagulation.*

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In 1936 Sterner and Medes[1] reported that cysteine and methionine prolonged both the bleeding and coagulation times for several hours when administered orally or intravenously to human subjects. They further concluded that the effect was mainly on one factor of the coagulation system, namely, prothrombin. From the data reported by these investigators one could conclude that methionine might possibly be an effective and much safer anticoagulant for clinical use than either dicoumarol or heparin. Because of these possibilities the effects of methionine on blood coagulation and quantitative prothrombin levels were studied more fully in human subjects.

In these studies dl-methionine was used. The solutions (2.5 g of methionine per 100 cc) for intravenous administration were made isotonic by the addition of sodium chloride and the pH adjusted to near neutrality by the use of sodium dibasic phosphate. The prothrombin determinations were done by the method of Quick[2] and the coagulation time by the intravenous test tube method of Lee and White.[3] Control studies were also done. All studies were done over a 6 hour period with observations at the initial 30 minute period and then each hour for the 6 hour period.

Control Studies. The coagulation times and prothrombin levels were determined in 3 persons in a fasting state over a 6 hour period. Changes were minimal. These studies were repeated in 3 persons after a routine hospital breakfast. Slight changes were noted in that the coagulation time was decreased after a period of from 4 to 5 hours, the maximum being 4½ minutes, this being within the range of experimental error.

Oral Methionine. Methionine was given to 2 individuals in 3.0 g amounts. No effect on the coagulation time was noted. No prothrombin determinations were made.

Intravenous Methionine. The individuals with normal prothrombin levels. Amounts of 1.3 g were administered to 2 subjects and no significant changes were noted in either the coagulation times or the prothrombin levels. Methionine in 2.5 g doses was given to 8 subjects, one fasting and 7 on routine hospital diet. In no instance was the coagulation time increased or the prothrombin level altered. In 4 individuals, including the fasting subject, the coagulation time was decreased, the maximum being a 10 minute decrease in one individual. The curve was a moderate exaggeration of the one obtained in the control subjects who had breakfast. Methionine in 5.0 g doses was administered to 3 subjects. No significant changes were noted in either the coagulation times or the prothrombin levels.

Intravenous Methionine. Individuals with decreased prothrombin. 2.5 g were administered to an individual with cirrhosis of the liver and observations made over a 6 hour period. The initial prothrombin level was

* This investigation was supported by a research grant from the National Institute of Health, U. S. Public Health Service.

1 Sterner, J. H., and Medes, G., *Am. J. Physiol.*, 1936, **117**, 92.

2 Quick, A. J., *J.A.M.A.*, 1938, **110**, 1658.

3 Lee, R. I., and White, P. D., 1913, **145**, 495.

35% of normal. No changes of any significance were noted in the coagulation times.

Conclusion. It has been previously reported that methionine prolonged the bleeding and coagulation times. In this study the effects of dl-methionine on the intravenous coagulation time and prothrombin were observed in human subjects. The changes were insignificant and dl-methionine has no clinical value as an anticoagulant.

16886

Vitamin B_6 Group. XV. Urinary Excretion of Pyridoxal, Pyridoxamine, Pyridoxine, and 4-Pyridoxic Acid in Human Subjects.*

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The earliest investigations of the excretion of vitamin B_6 by animals were made using the chlorimide reaction for analysis.[1,2] The method as then used was not specific for the determination of pyridoxine, but since the complex nature of vitamin B_6 was then unknown, all material found by this method was called *pyridoxine*. These early investigations did establish the fact that pyridoxine was rapidly absorbed from the digestive tract and rapidly cleared in the renal pathway. Although the recovery of the ingested vitamin in the rat was 50 to 70%,[1] only 10 to 20% of the dose was recovered when pyridoxine was fed to dogs or to human subjects.[2]

Subsequent refinement of the chlorimide method showed that small amounts of some substance other than pyridoxine were excreted in the urine of the dog and man after feeding a large dose of pyridoxine.[3] It was also shown that both pyridoxine and the unknown metabolite occurred in part as conjugated forms which did not react with 2, 6-dichloroquinone chlorimide, but which could be hydrolyzed with acid to give pyridoxine and the unknown metabolite.

The demonstration of the occurrence of *pseudopyridoxine* in human urine both before and after administration of pyridoxine[4] and the subsequent characterization of *pseudopyridoxine* as pyridoxal and pyridoxamine,[5] suggested the identity of the unknown metabolite of Scudi *et al.*[3] with pyridoxal or pyridoxamine.

The main metabolic product excreted after ingestion of pyridoxine was discovered by Huff and Perlzweig and identified as 4-pyridoxic acid.[6] This compound does not produce a color with the chlorimide reagent, and is inactive in promoting growth of microorganisms in vitamin B_6-free media.

Although pyridoxal and pyridoxamine are now known to be the forms of vitamin B_6 present in largest amounts in many foodstuffs and tissues,[7,8] no information is available concerning their metabolic fate. Development of a differential assay procedure for pyridoxal, pyridoxamine, and pyridoxine[8] makes such a study feasible. Results of such an investiga-

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

1 Scudi, J. V., Koones, H. F., and Keresztesy, J. C., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 118.

2 Scudi, J. V., Unna, K., and Antopol, W., *J. Biol. Chem.*, 1940, **135**, 371.

3 Scudi, J. V., Buhs, R. P., and Hood, D. B., *J. Biol. Chem.*, 1942, **142**, 323.

4 Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, 1942, **143**, 519.

5 Snell, E. E., *J.A.C.S.*, 1944, **66**, 2082.

6 Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1944, **155**, 345.

7 Snell, E. E., *J. Biol. Chem.*, 1945, **157**, 491.

8 Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, 1948, **176**, 1157.

tion conducted with normal human subjects are presented below.

Methods. Twenty-four hour samples of urine were collected from 3 adult male subjects and used as control samples to establish a base level for vitamin B_6 excretion by individuals on a normal diet. Individual subjects were then fed 100 mg of pyridoxine hydrochloride, pyridoxamine dihydrochloride, or pyridoxal hydrochloride in one dose with approximately 200 cc of water. Urine was collected 2, 5, 8, 12, 24, and 36 hours after ingestion of the test dose, and stored under toluene in amber bottles at 7° until analysis. Two months later the experiment was repeated using the same subjects but feeding each subject a different form of the vitamin than had been fed the first time.

The pyridoxal, pyridoxamine, and pyridoxine content of these samples was determined by the differential microbiological assay which uses *Lactobacillus casei, Streptococcus faecalis,* and *Saccharomyces carlsbergensis* as test organisms.[8] The pyridoxic acid content of the samples was determined by the fluorometric method of Huff and Perlzweig,[6] and is expressed in terms of the lactone.

Since a large part of the vitamin B_6 of natural materials is unavailable to the test microorganisms unless the samples are first autoclaved with acid,[9] control samples of urine were subjected to various conditions of acid hydrolysis to determine the optimal conditions for the liberation of vitamin B_6. The results (Table I) show that acid hydrolysis results in the liberation of increased amounts of vitamin B_6 in urine over that observed in unhydrolyzed samples. 0.055 N acid, the concentration commonly used for most samples[9,10,11] was found to be less effective than treatment with 0.11 N HCl or 0.55 N HCl. 0.11 N HCl was used instead of 0.55 N HCl in hydrolysis of the samples, since the amount of potassium chloride present in an assay tube after neutralization of samples treated with 0.55 N acid reaches approximately 135 mg per 5 cc of medium, which is close to the highest level of potassium chloride tolerated by *Saccharomyces carlsbergensis* and lactic acid bacteria.[12]

The samples collected were therefore treated in the following way prior to microbiological assay: 10 cc aliquots of each sample were autoclaved for 7 hours at 20 lb pressure in 180 cc of 0.11 N HCl. The samples were then neutralized with potassium hydroxide, diluted, and assayed.

TABLE I.
Liberation of Vitamin B_6 in Normal Urine by Acid Hydrolysis.

Treatment	Pyridoxal · HCl mγ/cc	Pyridoxamine · 2HCl mγ/cc
None	29	0
.055 N HCl*	78	133
.11 "	93	196
.55 "	93	210

* Autoclaved in 180 cc of acid at 20 lb pressure for 5 hr.

The limitations of the differential assay have been pointed out elsewhere,[8] especially in regard to the determination of the pyridoxine content of samples low in this form of vitamin B_6. In addition to these inherent limitations of the differential assay, it was found that the recovery of pyridoxine added to a normal urine sample was somewhat lower than could be expected from purely analytical errors.[8] Urine seemed to contain a material toxic for *Saccharomyces carlsbergensis* but not for *Streptococcus faecalis* or *Lactobacillus casei.* The presence of this toxic material results in an underestimation of whatever pyridoxine may be present in the control sample of urine, but does not affect the determination of pyridoxal or pyridoxamine in this sample. None of the results are affected in other samples, which contained much more vitamin B_6 and were therefore assayed at dilutions such that the toxic material was without effect.

Results. The distribution of the 4 compounds determined in normal urine is shown in columns *b* and *e*, Table II. The levels of

9 Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. and Eng. Chem.,* Anal. Ed., 1943, **15**, 141.

10 Rubin, S. J., Scheiner, J., and Hirschberg, E., *J. Biol. Chem.,* 1947, **167**, 599.

11 Rabinowitz, J. C., and Snell, E. E., *Anal. Chem.,* 1947, **19**, 277.

12 MacLeod, R. A., and Snell, E. E., *J. Biol. Chem.,* 1948, **176**, 39.

TABLE II.
The Urinary Excretion of Pyridoxic Acid, Pyridoxal, Pyridoxamine and Pyridoxine.

Excretion products*	Mg excreted up to 24 hr after ingestion (a)	Mg excreted 24 hr before ingestion (b)	Mg recovered (c)	Mg excreted up to 24 hr after ingestion (d)	Mg excreted 24 hr before ingestion (e)	Mg recovered (f)
	Fed 70 mg pyridoxamine. Subject A			Subject C		
4-pyridoxic acid lactone	24.60	6.48	18.12	22.37	2.59	19.78
Pyridoxal	1.57	0.102	1.47	0.985	0.058	0.93
Pyridoxamine	1.83	0.028	1.80	1.18	0.149	1.03
Pyridoxine	0.00	0.00	0.00	0.209	0.00	0.21
Total			21.39			21.95
	Fed 82 mg pyridoxine. Subject B			Subject A		
4-pyridoxic acid lactone	28.40	1.97	26.43	25.27	3.91	21.36
Pyridoxal	1.69	0.074	1.62	1.38	0.052	1.33
Pyridoxamine	0.535	0.124	0.41	0.650	0.210	0.44
Pyridoxine	8.53	0.00	8.53	7.80	0.00	7.80
Total			36.99			30.93
	Fed 82 mg pyridoxal. Subject C			Subject B		
4-pyridoxic acid lactone	59.79	3.84	55.95	53.22	2.54	50.68
Pyridoxal	1.53	0.084	1.45	1.28	0.049	1.23
Pyridoxamine	0.021	0.083	0.00	0.241	0.097	0.14
Pyridoxine	0.039	0.00	0.04	0.132	0.00	0.13
Total			57.44			52.18

* All products are expressed in terms of the free bases. Pyridoxic acid is determined and expressed as the lactone. The molecular weight of the free bases are almost equal (pyridoxal = 167, pyridoxamine = 168, pyridoxine = 169, 4-pyridoxic acid lactone = 165). Expressed in this way, weights are directly comparable as molar quantities without significant error.

these compounds were not particularly constant. Of the 4, pyridoxic acid was quantitatively the most prominent, accounting for from 91 to 98% of the total. Of the 3 forms of vitamin B_6, pyridoxamine was usually present in highest concentrations, although pyridoxal was sometimes present in higher concentrations. Some pyridoxal was always found in the urine. The presence of pyridoxine could not be demonstrated in any of the normal urine samples; but, as noted above, this result may be due to the limitations of the method of analysis and does not constitute satisfactory evidence that pyridoxine is not excreted under normal conditions.

When pyridoxamine was fed, a significant rise in the pyridoxal as well as in the pyridoxamine content of the urine was noted; in fact, the amount of the administered pyridoxamine recovered as pyridoxal was equal to the amount recovered as pyridoxamine. The amount of pyridoxine indicated as being formed was not significant. The principal excretion product was pyridoxic acid, which accounted for 85% of the measured excretion products for subject A and 90% for subject C.

After feeding pyridoxine, the major part of the vitamin B_6 excreted was in the form of pyridoxine, but 14 to 15% of the activity appeared as pyridoxal. The pyridoxamine of the urine accounted for only 4 to 5% of the vitamin B_6 activity of the urine. In this case 71 and 69% of the excretion products measured appeared as pyridoxic acid.

When pyridoxal was fed, larger amounts of pyridoxic acid were recovered than in either of the two preceding instances. However, the

total amount of vitamin B_6 in the urine was somewhat lower. 82 and 98% of this was pyridoxal.

Fig. 1 illustrates the fact that after feeding each of the 3 forms of vitamin B_6, a large increase in the level of the form fed occurs within 2 to 5 hours after administration of the

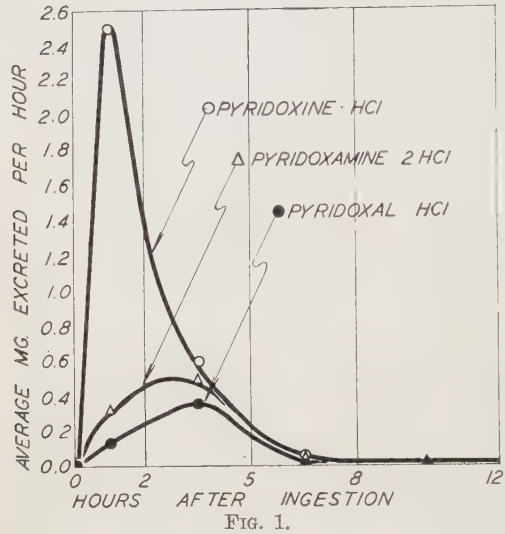

FIG. 1.

The Excretion of Vitamin B_6. The total amount of the indicated form of the vitamin found in the urine samples is plotted against the average time interval, after ingestion of the dose, during which the sample was collected.

○ Fed 100 mg of pyridoxine HCl.
△ Fed 100 mg of pyridoxamine 2HCl.
● Fed 100 mg of pyridoxal HCl.

The levels of the forms of the vitamin other than the one fed are not shown.

dose, and that this level returns to the normal value within 8 hours. Similar results, not shown in this graph, were also obtained in connection with the excretion of the forms of the vitamin other than the one fed. The pyridoxic acid levels of the urine, shown in Fig. 2, increase in a much more pronounced manner, and levels did not return to normal until 12 hours after ingestion of the dose.

The amount of the test dose recovered was characteristic of the form of the vitamin fed, judging from the agreement of the results obtained in the two experiments, in which different subjects were fed the same compound. The highest recovery of the test dose, 70% and 64%, was obtained after feeding pyridoxal. The amount of pyridoxine recovered as the 4 products measured was 45 and 38% while the smallest recovery, 31 and 31%, was obtained after feeding pyridoxamine. These results may be inferred from Fig. 2, since the major component of the excretion products is pyridoxic acid.

Discussion. The above comparison of the amount of the test dose recovered as excretion products is of interest, since with pyridoxine and pyridoxamine, less than half of the administered dose was recovered. With pyridoxal, the most highly oxidized form of the vitamin, the recovery was higher because of the increased excretion of 4-pyridoxic acid which resulted when this form was fed.

A number of explanations for these low recoveries might be postulated. It is possible, for example, that the absorption of the various forms of the vitamin from the tract is incomplete. This explanation, however, is not in accord with the observations of Scudi, *et al.*,[2] who showed with both dogs and human beings that the total urinary excretion of vitamin B_6 following administration of large doses of pyridoxine (50 to 100 mg) was closely similar whether the vitamin was given orally or by intravenous injection. This indicates rather conclusively that absorption of test doses of this size from the tract is complete. The amounts of "pyridoxine" (total chlorimide reacting substances) which they found excreted following ingestion of 100 mg of pyridoxine hydrochloride approximate closely the amount of vitamin B_6 (pyridoxal, pyridoxamine and pyridoxine) found in the urine of our subjects. When limited amounts of the three forms of the vitamin are fed to deficient rats, chicks, or dogs apart from the diet, they show equal growth-promoting activities,[13,14] again indicating equal absorption.

A second possible explanation for low recoveries would be that the ingested dose was being stored in the tissues. However, the subjects used were ingesting normal diets and thus were presumably not deficient in vitamin B_6. Consequently, no great storage of the ingested vitamin would be expected, and

[13] Sarma, P. S., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, **165**, 55.

[14] Sarma, P. S., Snell, E. E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 284.

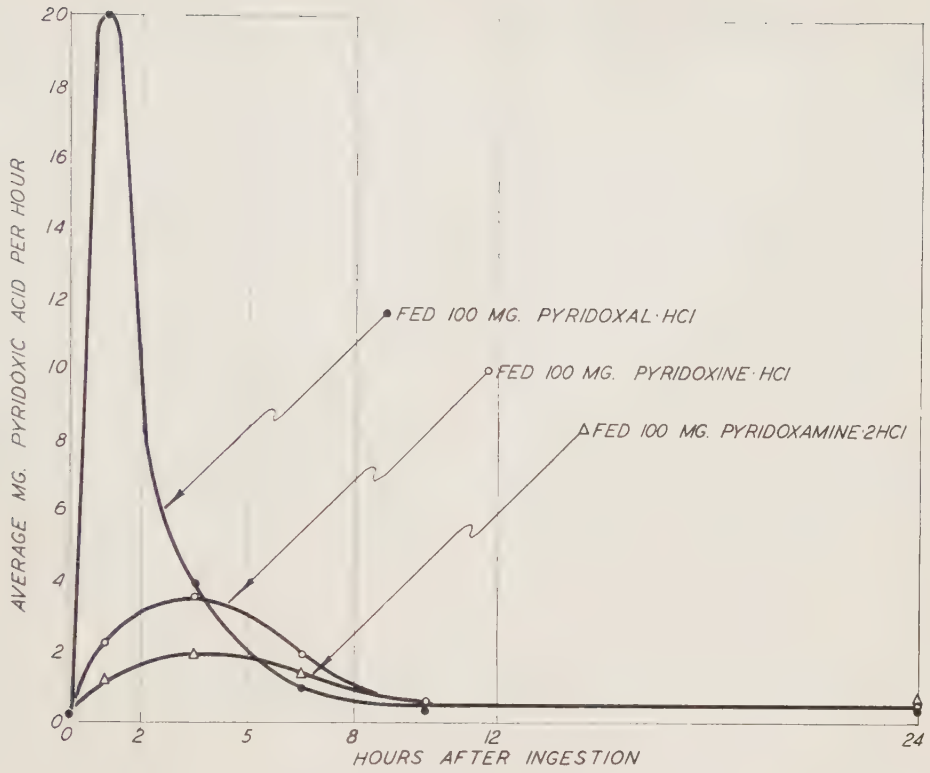

FIG. 2.

The excretion of pyridoxic acid (expressed as 4-pyridoxic acid lactone) following ingestion of various forms of vitamin B_6.

the fact that excretion levels rapidly returned to basal levels indicates that no great storage occurred. A third explanation for these low recoveries would be that still unidentified metabolic products which are without vitamin activity are formed from vitamin B_6 and excreted. From the considerations outlined above, this hypothesis seems most likely. It finds additional experimental support in the observation of Scudi, *et al.*[2] that following injection of 50 mg of pyridoxine hydrochloride in dogs, only 18% of this dose could be recovered in the urine. Yet, according to Huff and Perlzweig,[15] dogs do not excrete 4-pyridoxic acid. Unidentified excretion products, or complete oxidation within the body of considerable portions of the vitamin are thus indicated.

Pyridoxal is the only form of the vitamin which appears in the urine in greatly increased amounts as the result of feeding all 3 compounds. This further emphasizes the position of central importance indicated for this compound by direct assay of animal tissues,[8] by its universal availability to all microorganisms tested,[16] and by its occurrence in the only known catalytically active form of vitamin B_6, pyridoxal phosphate.[17]

Finally, it should perhaps be emphasized that the metabolic changes described above are those which occur when large amounts of vitamin B_6 are superimposed on a normal diet which already supplies the relatively small amounts of vitamin B_6 presumably required by man. Whether the small quantities normally ingested are metabolized in the same manner cannot be decided from these data.

Summary. The known metabolic products

15 Huff, J. W., and Perlzweig, W. A., *Science*, 1944, **100**, 55.

16 Snell, E. E., and Rannefeld, A. N., *J. Biol. Chem.*, 1945, **157**, 475.

17 Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, 1948, **161**, 311.

of vitamin B_6—pyridoxal, pyridoxamine, pyridoxine and pyridoxic acid—were measured in normal human urine and in the urine of human subjects each fed one of the 3 forms of the vitamin.

The chief product found, regardless of the form fed, was pyridoxic acid. Pyridoxal gave rise to significantly higher amounts of this product than did pyridoxine or pyridoxamine. No evidence could be obtained showing the conversion of pyridoxal or pyridoxamine to pyridoxine. When pyridoxal or pyridoxine was fed, the chief form in which the vitamin occurred in the urine was the form fed. However, when pyridoxamine was fed both pyridoxal and pyridoxamine were excreted in approximately equal amounts. Ingestion of pyridoxine also greatly increased the amount of pyridoxal and pyridoxamine excreted.

The excretion of all products was very rapid. The largest amounts of each of the compounds were found in samples collected 2 and 5 hours after ingestion of the dose. The levels of pyridoxic acid returned to normal values after 12 hours, while the vitamin levels had returned to normal within 8 hours. The amount of the dose recovered varied with the form fed. The highest recovery, 70%, was obtained when pyridoxal was fed; 45% of the pyridoxine was recovered, while only 31% of the pyridoxamine could be recovered. Together with published data which indicate that complete absorption of large doses of vitamin B_6 occurs, these findings suggest that a large proportion of the vitamin B_6 was converted to products still unknown.

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Loss of Body Protein and Antibody Production by Rats on Low Protein Diets.*

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(Introduced by Paul R. Cannon.)

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Previous studies in this laboratory have demonstrated that prolonged severe deficiency of protein without other dietary restriction results in a reduced capacity of the rat to produce antibodies to sheep erythrocytes,[1] Friedlander's bacillus,[2] pneumococci,[3] and most recently a parasitic nematode.[4] Concomitant with the reduction of antibody production there was shown to be a reduction in the capacity to form leukocytes of both the granulocytic and lymphocytic series.[5] Associated with these phenomena there is a reduction in the resistance of animals to infection with virulent organisms.[3] Furthermore this reduction in resistance was shown to be due largely to the inability of the animals to fabricate antibodies since the depleted animals, when passively immunized, survive the infection as well as the normally nourished controls. Having established the fact that protein depletion of long duration reduces the rate of antibody formation and the resistance to infection it then becomes of interest to investigate the rate of decay of the antibody forming capacity with time under conditions

* The research which this paper reports was undertaken in cooperation with the Navy Department, Office of Naval Research. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

The work has been aided, also, by the National Livestock and Meat Board and the Douglas Smith Foundation for Medical Research of The University of Chicago.

1 Cannon, P. R., Wissler, R. W., Woolridge, R. L., and Benditt, E. P., *Ann. Surg.*, 1944, **120**, 514.

2 Woolridge, R. L., unpublished observations.

3 Wissler, R. W., *J. Infect. Dis.*, 1947, **80**, 264.

4 Woolridge, R. L., unpublished observations.

5 Asirvadham, M., *J. Infect. Dis.*, 1948, **83**, 87.

of dietary protein deficiency and to attempt to relate this process to the loss of protein from various body compartments.

Materials and methods. The subjects of the experiments were young adult male albino rats (Sprague-Dawley). On arrival at the laboratory the animals were housed 7 to a cage and fed Wayne Dog Chow until they had reached a weight between 200 and 220 g. They were then started on the low protein regime. The detailed composition of the low protein diet (3E) has been described elsewhere.[6] The ration used in the present experiment contained corn oil as the fat. The content of all dietary essentials in this diet is sufficient to support normal growth when an adequate protein supplement is added. For the first 30 days of the depletion period the animals were offered 15 g of the ration each day. After 30 days the quantity was increased to 20 g per animal per day. Diet consumption is practically complete with this diet until more than 25% of the body weight has been lost after which diet intake slowly falls. The most depleted animals in this experiment never ate less than 72% (14.4 g) of their diet on the average.

In this study groups of 5 rats having comparable weights prior to depletion were selected from larger lots which had been on the depletion regime for 0, 17, 32, 43, 56, and 100 days. At the start of the final period of the experiment the animals were placed in individual cages and fed the same low protein diet for the last 11 days. On the day they were placed in the individual cages each animal was given by tail vein an injection of one ml of a 0.25% suspension of 5 times washed sheep's erythrocytes, and one ml of a heat killed vaccine of Friedlander's bacillus containing 1.6 billion organisms per milliliter. Six days after the injection of antigen, blood volumes, hematocrits, hemoglobins, serum protein concentrations and agglutinin and hemolysin titers were determined.[7] Five days following this the animals were sacrificed. The

TABLE I.* Protein Content Several Body Compartments and Rate of Antibody Formation in Rats on Low Protein Diet for Various Periods of Time.

Depletion to sacrifice, days	No. of animals	Live wt at sacrifice, g	Carcass protein, g	Total circulating serum protein, mg	Liver protein, g	Total circulating hemoglobin, mg	Depletion at time of inj., days	Agglutinin titers	Hemolysis titer
0	5	206	32.1		1.34				
		± 1.6	± .58		± .063				
11	5	177	30.8	514	1.07	2370	0	3840	7680
		± 2.1	± .33	± 19	± .038	± 36		± 0	± 0
28	5	171	31.0	410	1.25	1900	17	2200	6700
		± 2.3	± 0.22	± 7	± .067	± 94		± 300	± 940
43	5	169	30.3	390	0.91	1410	32	1280	1920
		± 3.7	± 0.60	± 10	± .047	± 79		± 220	± 0
54	5	161	30.7	380	0.84	1490	43	960	1920
		± 1.9	± 0.75	± 6	± .042	± 61		± 0	± 390
67	5	147	29.1	341	0.73	1290	56	320	1280
		± 1.4	± 0.60	± 12	± .021	± 61		± 90	± 370
111	4	129	23.2	266	0.70	1150	100	18	340
		± 4.6	± 1.00	± 15	± .041	± 69		± 3	± 70

* Values are given as means and standard errors for groups of animals.

[6] Wissler, R. W., Woolridge, R. L., Steffee, C. H., and Cannon, P. R., *J. Immunol.*, 1946, **52**, 267.

[7] Benditt, E. P., Straube, R. L., and Humphreys, E. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 189.

carcasses and livers were analyzed for fat, water and protein by methods which have been described.[8] Sections were made of the livers and these in conjunction with the detailed liver analyses are the subject of another report.[9]

Observations. The observations are summarized in Table I. In Fig. 1 are plotted the

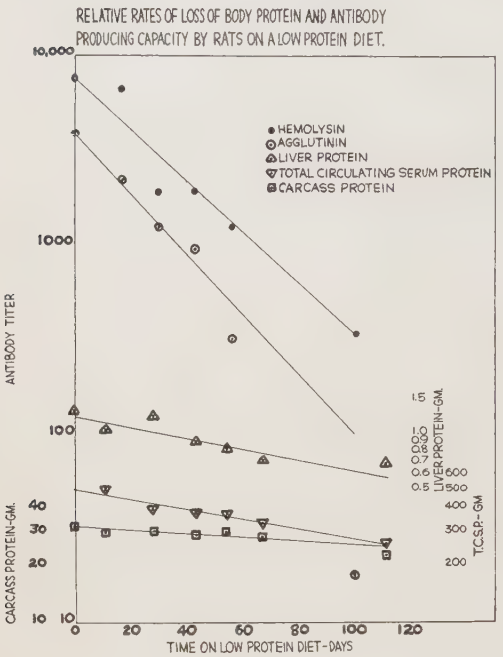

FIG. 1.

data for carcass protein, liver protein, total circulating serum protein, and the antibody titers (agglutinins and hemolysins). They are plotted on a semilogarithmic scale with time on the linear axis. Such a chart allows one to compare the relative rates of decline of these various factors by comparing the slopes of the curves.

To a reasonable first approximation the loss of protein from the carcass, liver, serum and erythrocytes occurs at a constant percentage rate. The same is true for the loss of the capacity to form antibody. The daily percentage rates of decline as estimated from these curves are: Carcass, 0.3; total circulating serum protein 0.6; liver protein, 0.7; rates of antibody production, antibacterial agglutinins), 3.7; and (anti-sheep erythrocyte hemolysin), 3.0. The rate of loss of hemoglobin is approximately the same as for serum protein. Liver and plasma proteins decline at approximately equal rates, and approximately twice as fast as the carcass protein. The antibody forming capacity declines 10-13 times as fast as the carcass protein content.

Statistical analysis shows that the decrease in hemolysin titer is not significant until after 30 days following initiation of the low protein diet. The agglutinin titers appear to be just significantly diminished ($p < .05$) by the 17th day of the depletion regime.

Discussion. Recently Metcoff and co-workers[10] have questioned the role of dietary protein deficiency in impairment of antibody formation. These investigators injected young rats with *S. typhimurium* 6 days after initiation of the low protein diet. As might be expected, they could find no difference in antibody titers up to 28 days from the start of the protein deficiency. On the 28th day they injected typhoid "O" antigen and 14 days later removed blood for titers in an attempt to demonstrate an "anamnestic" response. From their experience these authors conclude "that dietary protein deficiency does not seriously impair the mechanism for protective antibody formation in the rat."

The present observations demonstrate clearly that severe restriction of the dietary protein intake, despite the presence of an adequate supply of other essential nutrients causes marked impairment of antibody production. Further they demonstrate that the capacity to form antibody decreases with time on the low protein diet but the decrease does not become appreciable with some antigens until several weeks or more have elapsed. Whipple and his co-workers[11] early recognized that many weeks of protein depletion were

[8] Benditt, E. P., Humphreys, E. M., Wissler, R. W., Steffee, C. H., Frazier, L. E., and Cannon, P. R., *J. Lab. and Clin. Med.*, 1948, **33**, 257.

[9] Jaffe, E. R., Humphreys, E. M., Benditt, E. P., and Wissler, R. W., *Arch. Path.*, in press.

[10] Metcoff, J., Darling, D. B., Scanlon, M. H., and Stare, F. J., *J. Lab. and Clin. Med.*, 1948, **33**, 47.

[11] Madden, S. C., and Whipple, G. H., *Physiol. Rev.*, 1940, **20**, 194.

necessary to exhaust the "protein reserves" of their animals and reduce the rate of plasma protein fabrication significantly.

Since antibody formation appears to be influenced by dietary protein intake it seems likely in general that anything which interferes substantially with protein metabolism such as caloric inadequacy[8] for a sufficient length of time will also impair antibody formation.

One further point is apparent in the data of this experiment. The protein *content* of any body compartment is an insensitive indicator of the rate of turnover or fabrication of protein in the compartment. In Fig. 1 it can be seen that when the total circulating serum protein fell to about 50% of its initial value the rate of antibody production has fallen to 1% or less of its initial value. From previous experiments[12] we know that the circulating quantity of that portion of the globulin containing the antibody is reduced only to an equal or lesser extent than the total protein, *i.e.* 50% or less. Practically this means that the measurement of such quantities as serum protein concentration, total circulating serum protein or any one of its components may give little if any insight into the *rate* of production or turnover of the protein.

Summary. The present experiment investigated the rate of loss of the capacity to produce antibody in comparison with the rate of loss of protein from body compartments including carcass, liver and plasma. Young adult male albino rats were tested at intervals from 0 to 100 days after initiation of the low protein diet for agglutinin and hemolysin production. Blood volumes, hemoglobin, serum protein, liver and carcass protein were determined. It was found that all protein compartments and the rate of antibody production fell at approximately linear percentage rates per day, the antibody production falling much faster than the rest. Statistically significant depression of the antibody titers was not reached till the 30th day with hemolysins and the 17th day with the agglutinins.

12 Benditt, E. P., unpublished observations.

16888

Chemotherapy of *Trichomonas foetus* Infections in Rabbits.*

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No satisfactory therapeutic agent has been established for the successful treatment of bovine trichomoniasis. *In vivo* studies in laboratory animals of the trichomonacidal properties of various chemicals may offer useful information for future treatment trials in cattle. The purpose of this paper is to present the results of a study on the effect of 58 compounds on *Trichomonas foetus* in the vagina of rabbits. The majority of the compounds were selected from 350 chemicals previously tested *in vitro* by Morgan and Campbell.[1]

Witte[2] apparently was the first to successfully infect rabbits with *T. foetus* by vaginal

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† Wellcome Research Fellow of the Animal Health Trust (from the Ministry of Agriculture and Fisheries Laboratories, Weybridge, England).

1 Morgan, B. B., and Campbell, H. M., *Am. J. Vet. Res.*, 1946, **7**, 45.

2 Witte, J., *Arch. wiss. prakt. Tierheilk.*, 1933, **66**, 333.

inoculations. Trichomonads were recovered from the inoculated rabbits intermittently for 26 days. Nelson[3] was successful in infecting 2 rabbits intravaginally. According to Nelson[4] and Byrne and Nelson[5] 38 rabbits which were given intravaginal injections of *T. foetus*, 32 or 84% developed self-limiting infections with an average duration of 23 days. MacDonald *et al.*[6] inoculated 76 rabbits intravaginally with *T. foetus* with an incidence of 67%. Wittfogel,[7] Stableforth and Scorgie[8] and Trussell and McNutt[9] were unable to infect rabbits by the intravaginal inoculation of *T. foetus*.

Nelson[4] was the first to use the rabbit as a test animal for various therapeutic agents against *T. foetus*. Attempts to rid the organisms from the vagina of infected rabbits with 3 pentavalent and 1 trivalent arsenicals and sodium bismuthyl tartrate were unsuccessful.

Experimental procedure. Virgin female rabbits, of different breeds, obtained from various sources were used in the experiments. The animals were 6 to 8 months of age and weighed from 5 to 8 lb.

The bacteria-free strain of *T. foetus* used in this work was isolated by Morgan and Wisnicky.[10] Forty-eight to 96-hour-old cultures were used. The organisms were cultivated on a modification of Schneider's citrate; whole egg and defibrinated bovine blood slants overlaid with buffered saline citrate solution with 5% bovine serum. The number of trichomonads was determined by hemacytometer counts. A standard suspension of 3 million organisms per ml was used for inoculation purposes.

Rabbits were inoculated with 10 ml of a trichomonad suspension into the vagina by means of a sterile glass tube 7 inches long and ¼ inch in diameter. In the initial screening tests only 1 rabbit was used for each drug. A rubber adapter was attached to the free end of the glass tube and the trichomonads introduced by means of a glass syringe.

Microscopic identification of *T. foetus* was made from recently collected vaginal samples. A sterile wooden applicator stick with a cotton pledget attached was enclosed in a glass tube and inserted into the vagina. The glass tube was withdrawn, the swab rotated and a mucus sample obtained. Material adhering to the cotton was mixed with 0.7% saline; placed on a slide and examined.

Throughout the course of these studies the external genitalia of the rabbits were washed with a disinfectant prior to inoculation of infective material or collection of mucus samples. All rabbits were sampled prior to the experiments and the material plated on blood agar to determine the presence of bacteria. In a number of rabbits a gram negative rod was isolated in pure culture. Whether this bacterium was part of the normal flora of the vaginal tract of the rabbit could not be determined; however, its presence apparently had no effect on the course of the subsequent *T. foetus* infection.

Vaginal inoculation of *T. foetus* infected from 20 to 80% of the rabbits. The factors responsible for variation in susceptibility are not well understood. Microscopical examinations were made during the first 5 days following the infective inoculation and only those rabbits which showed a considerable number of organisms in the collected discharges were utilized in the experiments. Where the infection appeared to be mild during this time the rabbits were discarded. By making this selection the infection persisted in the 30 untreated controls for a minimum of 20 days.

Fifty-eight rabbits were treated with various compounds after showing an infection for 5 days. Three drugs which inhibited *T. foetus* were further tested utilizing 10 rabbits for each drug and 10 controls. Liquids and jellies were introduced in the vagina with a glass

[3] Nelson, P., *Arch. Path.*, 1937, **23**, 744.

[4] Nelson, P., unpublished thesis, Library, University of Wis., 1938.

[5] Byrne, H., and Nelson, P., *Arch. Path.*, 1939, **28**, 761.

[6] MacDonald, E. M., *et al.*, *J. Immunol.*, 1948, **59**, 295.

[7] Wittfogel, H., *Inaug. Diss. Hannover*, 1935, p. 68.

[8] Stableforth, A., and Scorgie, N., *Vet. Rec.*, 1937, **49**, 253.

[9] Trussell, R., and McNutt, S., *J. Infect. Dis.*, 1941, **49**, 453.

[10] Morgan, B. B., and Wisnicky, W., *J. Am. Vet. Med. Assn.*, 1942, **100**, 471.

TABLE I.

Therapeutic Agents Used for Treatment of Vaginal Infections in Rabbits Induced by *Trichomonas foetus*.

1. Phemerol (*p-tert*-Octylphenoxyethy-oxyethyldi-methyl benzyl-ammonium-chloride) (Liquid) (.01%)
2. Sulfaguanidine (Powder)
3. Povage (Silver chloride-thiourea complex salt + 3-chloro-4-hydroxy diphenyl + osmo-kaolin (Powder)
4. Vioform (5-chloro-7-iodo-8-hydroxyquinoline) (Powder)
5. Sodium bicarbonate (Liquid) (5%)
6. Carbarsone (p-Carbaminophenyl arsonic acid) (Powder)
7. Negatan (Polymerized disulphonic-dioxydimethyl-diphenylmethane acids) (Suppository 10% negatol)
8. Tyrothricin (Water soluble jelly) (.01%)
9. Ceepryn vaginal powder borated (Cetylpyridinium chloride (.5%) + boric acid + kaolin + dextrose) (Powder)
10. Entozon granulate (2,3-Dimethoxy-6-nitro-9-(*y*-diethylamino-*B*-hydroxypropylamino) acridine dihydrochloride + 2-orthoxy-6,9-diamino-acidrine lactate + amyl saccharine + sodium biborate) (Liquid) (2%)
11. Iodine (Tincture) (Liquid) (0.5%)
12. Lugol's Solution (Iodine (5 gr.) + potassium iodide (10 gr.) + water (100 cc)) (Liquid)
13. Trypaflavine (2,8-Diamino-10-methylacridinium chloride) (Liquid) (1%)
14. Proflavine dihydrochloride (3,6-diaminoacridine dihydrochloride) (Liquid) (3%)
15. Meta Cine Douche Powder (Citric acid + papain + lactose + methyl-salicylate + eucalyptol + menthol + chlorthymol) (Liquid) (3%)
16. Silver picrate (1% dispersed in kaolin) (Powder)
17. Zonite (Sodium hypochlorite + sodium hydroxide + sodium chloride) (Liquid) (3%)
18. Merpectogel (Phenylmercuric nitrate (1:24,000) + pectin jelly) (Jelly)
19. Caprokol (Hexylresorcinol 1:1000 + alcohol + water soluble jelly) (Jelly)
20. Chinosol (8-Hydroxyquinoline sulfate) (Liquid) (5%)
21. Amertan (5% tannic acid + merthiolate) (Jelly)
22. Gentian violet jelly-merthiolate (Jelly)
23. Lactic acid (Liquid) (5%)
24. Acetic acid (Liquid) (5%)
25. Penicillin 10,000 units per cc (total 100,000 units) (Liquid)
26. Streptomycin (1000 units per cc) (total 10,000 units) (Liquid)
27. Mercuric chloride (Liquid) (.01%)
28. Metaphen (4-nitro-anhydro-hydroxy-mercury-orthocresol (Metaphen 1:2500)) (Liquid) (.5%)
29. Copper sulfate (Liquid) (10%)
30. Floraquin (5,7-diiodo-8-hydroxy-quinoline (Diodoquin) + boric acid + lactose + anhydrose dextrose) (Powder)
31. Lysol (Cresylic acid + neutral soap + glycerine) (Liquid) (0.25%)
32. Sodium perborate (Liquid) (5%)
33. Phenylmercuric acetate (Liquid) (.01%)
34. Chiniofon (7-iodo-8-hydroxyquinoline-5-sulfonic acid) (Powder)
35. Picric acid (Liquid) (1%)
36. Pine Oil Disinfectant (Liquid) (5%)
37. Potassium permanganate (Liquid) (1%)
38. Hydroxyquinoline benzoate (Liquid) (5%)
39. Sulfo-merthiolate (Sodium p-ethylmercuri-thiophenylsulfonate) (Powder)
40. Malachite Green (Liquid) (1%)
41. Actidione (Liquid) (1%)
42. Subtilin (Liquid) (5%)
43. Bovoflavin-salbe German proprietary compound (Ointment)
44. Propylene glycol dipropionate (Suspension in water) (20%)
45. Sodium 3-(gamma-hydroxymercuri-*B*-methoxypropyl-4-hydroxybenzoate) (Liquid) (1%)
46. Negatan No. 34 (Solution) meta-cresol sulfonic acid + formaldehyde (Liquid)
47. Sulfo-merthiolate (sodium p-ethylmercuri-thiophenylsulfonate, Lilly) 1:1000 (Jelly)
48. Gentian violet 1% with merthiolate (sodium ethylmercuri thiosalicylate, Lilly) 1:1000 (Jelly)
49. Quinacrine hydrochloride (Powder)
50. Pantaquine (6-Methoxy-8-(5-isopropyl-amino-pentylamino) quinoline phosphate (Liquid) (10%)
51. Sodium recinoleate (Liquid) (5%)
52. Potassium dithioformate (Liquid) (5%)
53. Sodium isopropylsulfonate (Liquid) (5%)
54. Sodium hydrogen sulfasalicylate (Liquid) (5%)
55. Sodium diethyldithiocarbamate (Liquid) (5%)
56. 2 methoxy 6-chloro-9 (2'-diethyl-amino-4'-butylamino) acridine 2 HCl (5%)
57. 2-methoxy 6 chloro-9 (*a*-di-*n*-propylamino) acridine 2 HCl (Liquid) (5%)
58. 7-chloro-4-bis (dimethylamino-2-propylamino) quinoline (Liquid) (1%)

syringe (10 ml per animal). Powders were insufflated into the vagina with a small insufflator at the rate of ½ g per rabbit. After treatment the rabbits were examined at various intervals for 3 to 5 weeks and sacrificed after 6 to 8 weeks. The evaluation was based on one application of a given drug.

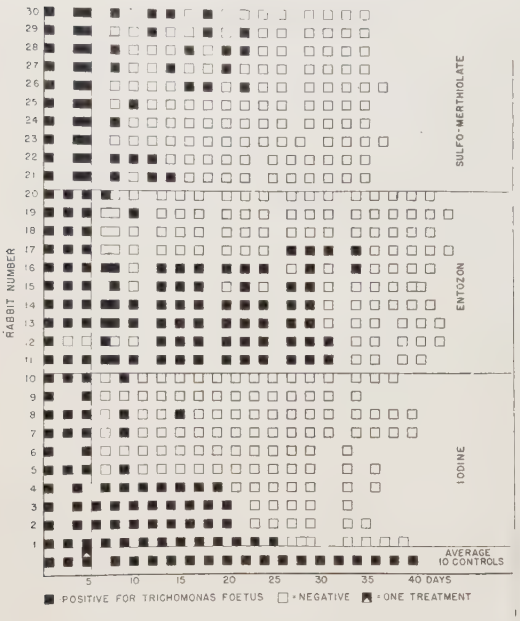

FIG. 1.

Results of *in vivo* tests with sulfo-merthiolate, entozon and iodine on *Trichomonas foetus;* 10 control rabbits.

Results. Table I indicates the 58 therapeutic agents used for treatment of the vaginal infections produced by *T. foetus;* of these compounds tested, only 3 appeared to reduce the duration of infection in the rabbits; entozon, iodine and sulfomerthiolate. Entozon, iodine and sulfo-merthiolate eliminated the infection, in 1, 4 and 5 days, respectively. The infection in all the other drugs tested persisted for at least 15 days following the treatment. None of the compounds tested at the dosage employed produced any symptoms of toxicity nor was there any apparent damage to the mucous membranes of the vagina on postmortem examination. The 3 drugs when used on 10 rabbits each appeared to shorten the duration of infection when compared with 10 untreated controls (Fig. 1). The results, however, are considered inconclusive.

Summary. A standardized, controlled *in vivo* technic utilizing female rabbits has been described for testing the trichomonacidal properties of various therapeutic compounds against *Trichomonas foetus.* A total of 58 compounds was tested by this method. Only 3 compounds: entozon, iodine and sulfomerthiolate appeared to reduce the duration of infection.

16889

Effect of Alpha Naphthylthiourea (ANTU) on Serum Cholesterol in Thyroidectomized Dogs.

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Chronic poisoning of dogs with alpha naphthylthiourea (ANTU) is accompanied by a marked increase of the cholesterol content of the plasma. After discontinuing administration of ANTU the plasma cholesterol diminishes rapidly.[1] ANTU like other thiourea derivatives depresses the function of the thyroid gland. This underfunction leads to compensatory enlargement of the gland. Richter[2] who introduced ANTU as a rodenticide described hyperplasia of the thyroid in rats poisoned with the drug. The antithyroid effect of the drug has also been demonstrated by cytological studies of the thyroid of rats

1 Chanutin, A., Gjessing, E. C., and Ludewig, S., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 174.

2 Richter, C. P., *J. Am. Med. Assn.,* 1945, **129**, 927.

TABLE I.
Total Serum Cholesterol in Mg Per Cent.

Thyroidectomy	Treatment	Minimum	Maximum	Mean	S.E.*	Ratio free: total cholesterol
Before	None	145	210	187	7	.27
After	Thyroid	74	225	165	10	.30
,,	Thyroid + ANTU	160	458	288	13	.33
,,	Thyroid†	150	294	221	25	.33
,,	Thyroid‡	177	210	196	6	.35

* Standard error of the mean.
† One week after discontinuing ANTU.
‡ Two weeks after discontinuing ANTU.

treated with ANTU.[3] Hypothyroidism is known to be accompanied by a rise in plasma cholesterol. The increase in plasma cholesterol in dogs chronically poisoned with ANTU could therefore possibly be due to the "chemical thyroidectomy", caused by ANTU.[1]

In order to determine the possible role of the thyroid, it was necessary to study the effect of ANTU poisoning on the serum cholesterol of thyroidectomized dogs. The thyroid deficiency due to removal of the thyroid had to be compensated by administration of desiccated thyroid by mouth. From studies in hypothyroid children[4] and thyroidectomized rabbits[5] it was known that the elevated serum cholesterol due to thyroid deficiency decreases rapidly to normal values under treatment with the thyroid hormone.

Material and methods. Six adult female dogs weighing around 14 kg were used. After a pre-operative control period the thyroid and parathyroid glands were removed surgically and the parathyroid glands reimplanted. Calcium lactate was given intravenously and by mouth during the first few days after operation. Feeding with Desiccated Thyroid U.S.P. 640 mg daily was started a week after operation. As soon as the dogs were stabilized on a normal cholesterol level feeding with ANTU (10 mg per kg bodyweight daily) was started. This was continued for a period of from 3 to 10 weeks. After this period ANTU was discontinued but treatment with desiccated thyroid was continued. In one of these dogs the chronic ANTU poisoning was repeated after the cholesterol level had reverted to normal for about 2 months. All dogs were sacrificed after completion of the study. No thyroid tissue or only minute amounts were found at autopsy.

Free and total cholesterol were determined by a modified Schoenheimer-Sperry procedure.[6] A number of determinations of total cholesterol were made without digitonine precipitation on samples extracted with alcohol-ether. The results for total cholesterol obtained by this method were in good agreement with those obtained by the modified Schoenheimer-Sperry procedure.

Results. A summary of our experiments is given in Table I. Both from this summary and from the individual data on each experiment it can be seen that the thyroidectomized dogs treated with adequate doses of thyroid show normal serum cholesterol levels. The cholesterol levels of the operated dogs treated with thyroid tend to be slightly lower than during the pre-operative period. This is in agreement with the known fact that serum cholesterol levels in the adequately treated hypothyroid patient are in the normal or low normal range. Chronic poisoning with ANTU superimposed on the treatment with thyroid hormone results in a marked rise in serum cholesterol in every experiment. After discontinuing ANTU without changing the dose

3 Jones, R. P., *J. Path. and Bact.*, 1946, **58**, 483.

4 Wilkins, L., and Fleischmann, W., *J. Am. Med. Assn.*, 1941, **116**, 2459.

5 Fleischmann, W., Shumacker, H. B., Jr., and Wilkins, L., *Am. J. Physiol.*, 1940, **131**, 317.

6 Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical Physiological Chemistry, 12th Ed. Blakiston Co., Philadelphia, 1945, p. 531.

of thyroid extract given by mouth the serum cholesterol level gradually reverts to normal values in every experiment. The ratio of free to esterified cholesterol stays within normal limits during the whole experiment and seems not to be appreciably changed by chronic ANTU poisoning.

Discussion. The experiments demonstrate that chronic poisoning with ANTU produces a reversible rise in serum cholesterol in thyroidectomized dogs treated with adequate doses of thyroid hormone. From our experiments it cannot be determined whether ANTU influences the metabolism of cholesterol or the distribution of cholesterol between blood and tissues. We can assume therefore, that ANTU produces a direct effect on serum cholesterol quite independent from an indirect effect due to the antithyroid properties of the drug.

The large dose of thyroid hormone used (640 mg daily) without inducing signs or symptoms of hyperthyroidism reflects the tolerance of dogs to large doses of thyroid hormone. The dog, in proportion to its body weight, has apparently a much greater capacity than does man to inactivate exogenous thyroid hormone. This inactivation seems to take place in the extrathyroid tissues. Therefore both normal and thyroidectomized dogs can tolerate large doses of thyroid hormone without damage.[7]

Summary. Chronic poisoning with alpha naphthylthiourea (ANTU) produces a reversible rise in serum cholesterol in thyroidectomized dogs maintained on a dose of thyroid hormone adequate to prevent thyroid deficiency. This indicates that the effect of ANTU on serum cholesterol is at least partly independent from its property as an antithyroid drug.

We wish to thank Mrs. Aurora Bradford for her assistance in the cholesterol determinations, and Miss Frieda Faiman for statistical analysis of the data.

[7] Danowski, T. S., Man, E. B., and Winkler, A. W., *Endocrinology*, 1946, **38**, 230.

16890

On the Chemical Sterilization of Blood and Blood Plasma.*

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The present study was undertaken in a search for a suitable means of chemically sterilizing human plasma and whole blood without so altering the blood components as to make them unsuitable for intravenous injection. The high incidence of homologous serum jaundice (4.5 to 7.2%) resulting from the injection of pooled plasma has become a serious problem in the use of pooled lyophylized plasma. At the present time only those methods involving the irradiation of plasma with ultraviolet light,[1] x-ray, or high speed electrons[2] have offered promise of a solution to this problem. Of these methods, only electron bombardment has appeared to offer any promise in the sterilization of whole blood. All of these irradiation technics require the use of highly specialized equipment which precludes the possibility of treating plasma or blood except at specially equipped plants.

The specifications for a chemical sterilizing agent are dictated by the future intra-

* Part of this material was presented at the Regional Meeting of the American College of Physicians, Detroit, Michigan, November 20, 1948.

† The authors are indebted to Dr. Elizabeth M. Yagle for the immunological data in this report.

[1] Wolf, A. M., Mason, J., Fitzpatrick, J., Schwartz, O., and Levinson, O., *J.A.M.A.*, 1947, **136**, 476.

[2] Trump, J. G., personal communication.

venous use of the plasma or whole blood. (1) It must not alter the plasma proteins in such a way as to make them toxic or antigenic. (2) The agent used must be nontoxic or must readily be converted to a nontoxic substance on standing or on suitable neutralization with a second nontoxic substance; (3) It should cause minimal or no changes in the immunological components of the blood; (4) It should not cause hemolysis nor increased fragility of red blood cells. In the present investigation, a member of the nitrogen mustard group, methyl-bis (beta-chloroethyl) amine hydrochloride has been chosen for study for a number of reasons. (a) This compound has been extensively studied as a chemotherapeutic agent against the leukemias and is available in purified form;[3] (b) Its cytotoxic action is believed to be due to its effect on nucleoproteins toward which it has been shown to react competitively to a marked degree;[4-7] (c) In buffered aqueous solution the compound readily hydrolyzes to form relatively nontoxic endproducts; (d) The mechanism of action of this substance on biological systems closely parallels that of ionizing radiations.

Experimental. I. Virucidal and Bactericidal Action of HN2. In the virus experiments a New Jersey strain of vesicular stomatitis virus was used.‡ The mice used were 3- to 4-week-old Swiss strain. After several mouse passages in our laboratory, the LD_{50} of the virus was found to be 0.03 ml intracerebrally of a $10^{-6.8}$ dilution. A 10% mouse brain suspension was prepared in rabbit serum, distributed to ampules, and frozen and stored in liquid oxygen for subsequent experiments. The inoculum was always 0.03 ml intracerebrally in mice and the titer of

TABLE I. Virucidal Action of HN2 in Human Serum, Plasma, and Whole Blood.

	Description % virus suspension	Time interval, days	LD/50 control	(D) Dosage mg/l vs. Mortality (M)						Final pH
				D	M	D	M	D	M	
1.	1% in 95% serum		10-5.2	500	3/8*					8.4
	same plus 2% 0.15 M $NaHCO_3$	5		500	3/8					8.5
	same plus 5% 0.15 M $NaHCO_3$	5		500	7/8					8.6
						200				
2.	1% in 95% serum	5	10-5.2	200	6/8	200†	8/8	400	6/8	500	3/8	7.9 to 8.4
				600	0/8	800	0/8	1000	0/8			
				400	5/5	500	5/5	600	3/5	800	3/5	8.0 to 8.2
		2	—	400	5/5	500	5/5	600	3/5	800	3/5	8.0 to 8.2
		5	—	400	5/5	500	5/5	600	3/5	800	3/5	8.0 to 8.2
3.	5% in 95% serum	2	—	400	5/5	500	5/5	600	3/5	800	3/5	8.0 to 8.2
		5	—	400	5/5	500	5/5	600	3/5	800	3/5	8.0 to 8.2
4.	2% in 100% serum	3	10-5.7	500	8/20	550	1/20	600	0/20	700	0/20	7.7 to 8.2
5.	1% in 98% citrated plasma‡	3	10-6.8	400	0/10	500	0/10	550	0/10	600	0/10	7.0 to 7.03
6.	1% in 98% citrated plasma‡	3	10-6.5	250	1/4	300	0/5	350	0/4	400	0/5	6.6 to 7.05
7.	1% in 90% citrated blood‡	3	10-5.8	400	1/5§	500	0/5	550	0/5	600	0/5	6.67 to 6.73
8.	1% in 90% citrated blood‡	3	10-6.6	250	3/5	300	2/5	350	0/4	400	0/4	6.68 to 6.85

* Denominator denotes number of mice tested, numerator denotes number of deaths.
† Second 200 mg/l added after 24 hr.
‡ In citrated blood and plasma experiments, enough $CaCl_2$ was added to remove the citrate, in this case 0.01 cc of 5 M $CaCl_2$ per cc of blood and plasma. Also enough heparin to prevent clotting once citrate was removed. The addition was made just before inoculation of animals.
§ Original bottle had to be diluted 1:10 because of excess of $CaCl_2$ which was toxic to the mice.

[3] Gilman, A., and Philips, F. S., *Science*, 1946, **103**, 409.

[4] Tenbroeck, C., and Herriott, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 271.

[5] Rose, H. M., and Gellhorn, A., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 83.

[6] Fruton, J. S., Stein, W. H., Stahman, M. A., and Golumbic, C., *J. Org. Chem.*, 1946, **11**, 571.

[7] Gjessing, E. C., and Chanutin, A., *Cancer Research*, 1946, **6**, 593.

‡ Received from Dr. Carl E. Duffy.

the control sample of virus was never less than 10^{-5}, even after standing for 5 days at 4°C. In each experiment the stock virus was added to the medium desired to make a concentration of 1 to 5% brain, the HN2 added in saline immediately after dissolving, and the mixtures and controls allowed to stand for 3 to 5 days in the icebox to allow the HN2 to react with the virus and the excess HN2 to hydrolyze. The material was then tested by inoculation in mice and any questionable deaths decided by passage of the mouse brain.

The results of studies on the virucidal action of HN2 in the presence of serum, ACD citrated plasma, and whole blood are presented in Table I. A consistent effect of pH has been observed, with more favorable results at pH values of 6.7 to 7.2 than at higher pH values. Destruction of the stomatitis virus is complete in ACD citrated plasma at 300 mg/l and in ACD citrated whole blood at 500 mg/l, while sterilization in serum required from 250 to more than 800 mg/l depending upon the pH and amount of serum added.

The bactericidal effects of HN2 have been studied on several organisms. In the absence of plasma, *B. coli* is killed by dilutions of HN2 as low as 100 mg/l. In inactivated citrated plasma at pH 7.1, the sterilizing dosage of HN2 is 450 mg/l after storage of the treated material 5 days in the icebox. Under the same conditions hemolytic streptococcus was killed at 450 mg/l, *Staphylococcus aureus* (strain 209 P) at 800 mg/l and *Pseudomonas aeruginosa* at 350 mg/l. Each organism was treated with HN2 at increments of 50 mg/l of HN2 up to 500, and increments of 100 mg/l up to 1200. 0.1 ml aliquots were used in each instance for subculturing and the treated tubes and controls were made up to contain initially about 1 million organisms per ml. A study of the time course of destruction of *B. coli* showed that the organisms were progressively destroyed over a 3 day period during which the active vesicant is present. At the end of this time the organisms will resume growth at 37° in the treated samples if not entirely killed.

II. Toxicity of HN2 treated Blood and Plasma. The toxicity of freshly dissolved HN2 has been extensively investigated and is of the order of about 3 mg/kg to various animals by the intravenous route. If allowed to stand in unbuffered solution there is an accumulation of the ethylenimonium ions which under certain circumstances may increase the toxicity beyond the original value due to the greater neurotoxic action of these intermediates.[9]

In bicarbonate buffer at pH 8.0 or in biological systems containing adequate buffering power to permit the degradation of the ethylenimonium ion at the end of 3 days at room temperature the chloroethyl and ethylenimonium compounds have disappeared and the toxicity decreased to the order of one thousandth its original value.[8] In the present experiments it has been found that appreciable toxicity may remain at 3 days in preparation stored in the refrigerator at 4-10°C, but after 5 days mixtures of blood, plasma or serum containing up to 1000 mg/l of HN2 are entirely innocuous to mice. Dosages as high as 5% of the body weight intravenously or 15% of the body weight intraperitoneally of citrated plasma containing 600 mg/l of HN2 hydrochloride are tolerated by mice without evidence of unfavorable reactions if the citrate effect is avoided with calcium chloride. The marked slowing of the decomposition by low pH values should, however, be emphasized.

Further evidence of the harmless nature of the end products of hydrolysis of HN2 in blood and plasma and of the probable lack of serious antigenic responses have been obtained in experiments on dogs and man. HN2 treated citrated plasma was administered to a dog weighing 18½ lb at 3 to 6 day periods as follows: 6 doses of 100 ml of plasma treated with 100 mg/l; twice with 100 ml of plasma containing 250 mg/l; once with 50 ml containing 2000 mg/l; twice with 100 ml containing 600 mg/l. Another dog initially weighed 23½ lbs and was intravenously ad-

[8] Golumbic, C., Fruton, J. S., and Bergmann, M., *J. Org. Chem.*, 1946, **11**, 518.

[9] Golumbic, C., and Bergmann, M., *J. Org. Chem.*, 1946, **11**, 536.

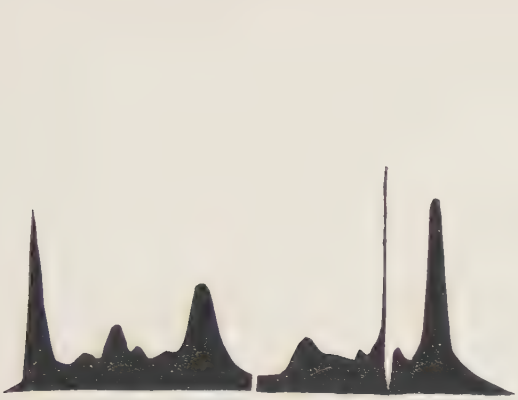

FIG. 1a.
Electrophoretic pattern of citrated plasma treated with 250 mg/l of HN2. (Barbital buffer, pH 8.6, μ 2 0.01.)

FIG. 1-b.
Electrophoretic pattern of control plasma from same sample.

ministered HN2 treated citrated whole blood as follows: 6 times with 100 ml of blood treated with 100 mg/1; once with 100 ml of blood containing 250 mg/1. Both animals remained in excellent condition throughout and following the treatment period. Their final weights following the last injection were 21 and 25¼ lbs respectively.

One normal individual has received a total of 5 doses of HN2 treated (600 mg/1) citrated type O plasma at approximately 2 week intervals as follows: 0.5 ml intradermally, 10 ml subcutaneously, 20 ml intravenously, 250 ml intravenously, and 250 ml intravenously. A second individual who has become locally sensitized to the unreacted beta-chloroethyl vesicant by repeated contact has received several intradermal, subcutaneous and intravenous injections of the same material without any evidence of sensitivity to the reaction products of the HN2 and plasma. No detectable reactions of any type have followed any of these tests. They clearly demonstrate the harmless nature of the final mixtures (10 days after mixing) and suggest that antigenic reactions are not likely to occur.

III. Biochemical Studies. Equally important with the virucidal activity and the nontoxic nature of the blood or plasma-HN2 reaction product are the alterations which occur in the plasma proteins and the red blood cells. A series of studies was, therefore, undertaken to ascertain the degree of alteration of some well known plasma proteins and of red blood cell properties and constituents.

A. Plasma Constituents: The extent to which added HN2 appears in the protein free filtrate has been roughly estimated by measurement of the amount of nitrogen in the protein free filtrate before and after the addition of various amounts of HN2 hydrochloride.

Most of the added HN2 appears in the protein free filtrate fifteen minutes after its addition while at 2 days and 5 days less than half of the added nitrogen is still present. The possibility of a part of the HN2 reacting with both NPN constituents and protein via the 2 available ethylenimonium groups should not be disregarded. Urea nitrogen determinations showed that no urea as measured by the urease method had disappeared.

The effect of HN2 on the A/G ratio has been determined. No measurable effect of 1000 mg/1 could be demonstrated 10 days later, while a marked difference was evident between samples stored at room temperature and those which were refrigerated. Additional evidence of the minor nature of the changes is seen in the electrophoretic pattern data prepared through the courtesy of Dr. Charles Janeway and Dr. John Newell, Massachusetts Blood Center, presented in Fig. 1. Visually the pattern exhibited a slight broadening of the albumin peak and increase of the ϵ-globulin peak.

The plasma fibrinogen concentration has

TABLE II.
Effect of HN2 on Prothrombin in Acid Citrated Whole Blood at pH 6.7-6.8 Three Days After Treatment.

HN2, mg/l	Prothrombin, % fresh control	HN2, mg/l	Prothrombin, % fresh control
Control	82	650	17
Control with HCl eq. to 1000 mg/l	74	700	15
450	38	750	12
500	16	800	10
550	22	900	9
600	16	1000	6

TABLE III.
Effect of HN2 on Plasma Prothrombin of Citrated Plasma and Whole Blood Treated with 600 mg/l of HN2.

Hours	Prothrombin, % of normal control		
	Plasma	Whole blood*	Whole blood†
1	54	80	—
1½	60	66	66
3	46	46	50
20	42	46	46
27	37	46	46
44	37	42	46
51	37	40	40
68	34	44	48
77	34	52	52
92	34	44	52

* Plasma separated after one-half hour.
† Separated just before each prothrombin determination.

been measured before and after treatment with HN2. No decrease is produced by concentrations up to 1000 mg/l. Clot formation progressively fails with the addition of higher concentrations (complete inhibition at about 1%) even in the presence of large excesses of added thrombin.

Of all the substances studied to date, only prothrombin time (Quick's method) is markedly affected by the sterilizing dosage of HN2. Table II shows the effect of HN2 on apparent prothrombin activity and Table III shows the time course of the inactivation of prothrombin in plasma, whole blood, and whole blood separated one hour after addition of HN2. Whether the apparent inactivation of prothrombin is due to the effect of HN2 on prothrombin or accelerator globulin is being investigated.

Other substances studied to date included alkaline phosphatase, complement and immune bodies. None of these substances were markedly affected at 500 mg/l of HN2.

B. Effect of HN2 on Red Blood Cells. The possible use of chemical sterilizing agents in whole blood is predicated upon the ability of the chemical used to destroy the contaminating organisms without a concomitant destruction of the red blood cells or without so altering them as to render them short lived when introduced into the body. At the present time the *in vivo* survival of the red cells has not been adequately investigated but such studies have already been arranged.

Numerous samples of citrated whole blood have been treated with HN2 during the various studies carried out to date. Somewhat different results have been obtained on the 3 species studied. Human blood in ACD citrate-glucose solution have uniformly exhibited good storage qualities over periods up to 3 months as compared with the control bloods. In no case with dosages of HN2 hydrochloride of up to 800 mg/l have the treated specimens shown any evidence of deterioration greater than that of the same blood control. We have, in fact, sometimes observed that the treated bloods failed to exhibit hemolysis until several days after the untreated controls showed definite hemolysis. At dos-

TABLE IV.
Effect of HN2 on the Rate of Hemolysis of Red Blood Cells.

Dosage of NH2, mg/l	$NaHCO_3$	pH	Hemoglobin determined as oxyhemoglobin, g/100 ml of plasma	Hemoglobin determined as cyanmethemoglobin, g/100 ml of plasma
Control	No	6.70	1.28	1.30
450	"	6.63	1.46	1.50
600	"	6.69	1.40	1.38
800	"	6.67	1.39	1.40
1000	"	6.62	1.32	1.37
450	Yes	6.71	1.18	1.18
600	"	6.70	1.13	1.15
800	"	6.78	1.10	1.12
1000	"	6.73	1.14	1.17

(The ACD citrated blood was treated one hour after collection with the amount of HN2 indicated, with or without the calculated amount of $NaHCO_3$ to neutralize all 3 chlorides, stored without disturbing for 3 months, thoroughly mixed, centrifuged, and the hemoglobin determined in the supernatant plasma.)

ages of 450-600 mg per liter, careful studies have shown that the onset and rate of hemolysis of refrigerated whole blood is approximately equal in treated and untreated blood, if pH is adequately controlled. Observations on citrated human blood and rabbit blood have indicated that these are not affected by the sterilizing dosages of HN2. Dog blood, however, will not tolerate dosages of HN2 in excess of 250 mg/l. At 600 mg/l, the onset of hemolysis is rapid (within 24 hours) and hemolysis is almost complete within a week. This is in marked contrast to the behavior of human blood which remains in apparent good condition at the end of one week after treatment with as much as 1500 mg/l of HN2.

The results of application of various doses of HN2 upon red blood cell fragility has been investigated. Very little change was observed in fragility at sterilizing doses of HN2, but the cells became increasingly susceptible to hypotonic salt solutions as the dosage approached 1000 mg/l.

Discussion. It has been demonstrated that HN2 is capable of exerting a bactericidal and virucidal action in whole blood, blood plasma and blood serum without causing major alterations in the properties of either the plasma components or the red blood cells. These observations are supported by the findings of Tenbroeck and Herriott[3] and Rose and Gellhorn[4] on the virucidal action of this compound on 5 other viruses in the absence of blood. Because of the present inability to transmit to animals the agent of virus hepatitis, it has not been possible to study this virus until careful studies had been made upon the characteristics of treated plasma. It is felt that the present evidence is sufficiently strong to warrant the direct study of this virus as soon as a more extensive group of recipients have received the treated plasma.

The data presented in this paper strongly suggest that at least two variables are involved in the virucidal activity of HN2—the competition of the virus with plasma and whole blood components, and the effect of pH. Much of the effect of varying the percentage of plasma or serum on the virucidal activity of HN2 has now been traced to the buffering action of the blood. In contrast to the supposed advantage of a pH of about 8.0, it has been observed that virucidal action is greatly enhanced by decreasing the pH to 7.2 or below. It seems likely that the beneficial effect of a lower pH is due to a reduction in the rate at which HN2 decomposes and/or reacts with other competing substances.

On the basis of studies carried out to date, it seems likely that the required sterilizing dosage of HN2 will be no greater than 500 mg/l if the pH of the treated material is held at or below 7.2. ACD citrated blood pH values range from 6.7 to 7.0. Studies are now under way to establish the optimum pH for the virucidal and bactericidal action of HN2.

Summary. Methyl-bis (beta-chloroethyl) amine hydrochloride has been demonstrated to exert effective virucidal and bactericidal effects in the presence of either plasma, serum

or whole blood. The required virucidal dosage of HN2 is below 500 mg/l of blood or plasma if the pH is at or near 7.0. The end products of decomposition of the added HN2 are essentially nontoxic if the pH of the system is not too low. No evidence of antigenic or other toxic reactions has been produced in two dogs and two humans receiving repeated injections of treated plasma. Human red blood cells withstand the application of virucidal dosages of HN2 without hemolysis and with only a slight increase in fragility. Complement, immune bodies, phosphatase and fibrinogen are only slightly affected by sterilizing dosages of HN2. Prothrombin time is markedly prolonged by HN2.

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Timed Intravenous Infusion of Metrazol and Strychnine for Testing Anticonvulsant Drugs.*

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The validity of the use of metrazol for the evaluation of anticonvulsant drugs has been well established.[1,2] When given to animals, subconvulsant doses of metrazol will elicit electro-encephalographic patterns similar to those found in patients with petit mal.[3] The marked antagonism between metrazol and 3,5,5-trimethyloxazolidine-2,4-dione (Tridione) and the success of this drug in the treatment of petit mal epilepsy further substantiate the validity of the metrazol antagonism test as a method for screening anti-epileptic compounds.

The present metrazol test method employs the subcutaneous route of administration whereby an effective dose of the anticonvulsant is injected into a group of animals, followed, after time is allowed for absorption, by a subcutaneous dose (usually 90 mg/kg) of metrazol known to be convulsant in the non-protected animal. If the animals fail to have seizures, a second group is given a higher dose of metrazol. This procedure is repeated until a dose is attained which produces typical convulsions. In this way the degree of protection (or metrazol antagonism) furnished by a drug in question may be ascertained.

For several years we have used a timed intravenous infusion of metrazol in mice, and have found it to have some distinct advantages over the previous procedure. A similar procedure has been evolved for the measurement of strychnine antagonism by anticonvulsant drugs.

Method. The apparatus employed consists of a cone-shaped mouse holder made of plexiglass and attached to a metal base. The plexi-glass makes it possible to clearly observe the reactions of the animal within the holder. A slit, through which the tail of the mouse is pulled, extends the length of the cone at its top. Attached to the base is a small electric light connected in series through a transformer to a synchronous electrical timer. The synchronous timer is set so that the electric bulb lights every 10 seconds. The reflection of the light is transmitted throughout the transparent holder and thus is readily noted by the observer.

The mouse is pulled into the holder by drawing its tail through the slit in the cone, and the injection is made into a tail vein, using a 27 gauge needle. A 0.5% solution of metrazol or a 0.01% solution of strychnine sulfate is employed in all tests, and injected

* This study was subsidized in part by grants from the Bristol Laboratories and the Mallinckrodt Chemical Works.

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at the rate of 0.05 cc every 10 seconds.

The reactions of the mouse to the timed intravenous infusion of metrazol follow a definite pattern. Three "signs" of reaction follow each other in strict sequence as the concentration of metrazol is increased in the blood stream. The first of these is the "first twitch," a sharp single twitching of the animal's entire body. This is followed very shortly by a "pseudoconvulsion," a series of clonic movements usually accompanied by an audible squeak. Very often the mouse pulls his head down under his body. The "pseudoconvulsion" is followed by alternate clonic movements and resting phases. The third sign, or "persistent convulsion," appears at the highest concentration of metrazol, and is the final end-point of the injection. The "persistent convulsion" consists of a tonic flexor component followed by a usually lethal tonic extensor component.

The doses of a 0.5% solution of metrazol at which the above-mentioned reactions occur were determined in control animals. Results appear for groups made up of mice weighing 15-17 g, and another series weighing 18-20 g (Table I). It will be noted that the thresholds of the mice weighing 15-17 g are lower, but that the mg/kg dosage is approximately the same.

In determining the efficacy of an anticonvulsant drug, observations were made on the ability of the compound to elevate the "first twitch," "pseudoconvulsion," and "persistent convulsion" thresholds. Also, the ability of a compound to protect the animals from death and to modify the severity of the clonic convulsions was considered.

The intravenous infusion of strychnine was likewise undertaken because of the chemical similarity of phenurone (phenyl acetyl urea) to myanesin (ortho-tolyl glycerol ether) and the fact that clinically a few patients report muscular weakness as a side effect of phenurone therapy.[4] Berger and Bradley[5] have shown that myanesin protects against strychnine convulsions. From our data this test

TABLE I. Intravenous Metrazol Infusion—Mice.

Groups	No. mice	1st twitch, cc	Pseudoconvul., cc	Persistent convulsion, cc	Type of seizure	Mortality, %	Time of death, min.
Normal, 15-17 g	40	0.14 ± .027 (1.00)	0.17 ± .037 (1.00)	0.45 ± .104 (1.00)	Tonic, flex.-ext.	100	Immed.
" 18-20 g	20	0.175 ± .026 (1.00)	0.23 ± .061 (1.00)	0.55 ± .181 (1.00)	" "	100	"
Tridione, 500 mg/kg 1 hr	20	0.37 ± .060 (2.64)*	0.44 ± .099 (2.57)	0.86 ± .003 (1.90)	" "	100	"
" 500 mg/kg 1½ hr	20	0.33 ± .058 (2.36)	0.40 ± .097 (2.37)	0.76 ± .109 (1.67)	" "	100	"
Phenurone, 400 mg/kg 1½ hr	20	0.32 ± .037 (2.29)	0.44 ± .067 (2.60)	0.83 ± .108 (1.83)	Clonic	30	82
Phenobarbital, 50 mg/kg 2 hr	20	0.36 ± .050 (2.55)	0.44 ± .062 (2.60)	0.85 ± .080 (1.89)	"	100	7.6
Myanesin, 300 mg/kg 10 min.	20	0.24 ± .043 (1.38)	0.32 ± .054 (1.39)	0.61 ± .088 (1.1)	"	100	27

* = Ratio: $\frac{\text{Threshold after treatment}}{\text{Normal threshold of corresponding wt group.}}$

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[5] Berger, F. M., and Bradley, W., *Brit. J. Pharm. and Chem.*, 1946, **1**, 269.

TABLE II.
Intravenous and Subcutaneous Metrazol in Mice.

	Intravenous							
	Control group—80 mice			Tridione 500 mg/kg I.P. 1 hr—20 mice				
	Dose mg/kg	S.D. mg/kg	S.E. mg/kg	Dose mg/kg	S.D. mg/kg	S.E. mg/kg	No. of S.D. above control threshold	Ratio: Tridione/control
First twitch	42	7	.8	111	19	4	9.9	2.6
Pseudoconvul.	51	13	1.4	131	32	7	6.2	2.6
Persistent convul. and death	134	36	4	259	38	8.5	3.5	1.9
	Subcutaneous							
	80 mice			50 mice				
CD-50	55	8	1.7	124	37	6.7	8.6	2.3
CD-95	62			178				2.9
LD-50	99	17	2.7	169	25	5.5	4.1	1.7
LD-95	121			200				1.7

may further elucidate the site of action of potent anticonvulsants.

The apparatus and technique are essentially the same as the metrazol method. A 0.01% solution of strychnine is injected at the rate of 0.05 cc every 10 seconds in all tests, after time has been allowed for absorption of the compound in question. The endpoint is the tonic extension of the animal's hind legs, *i.e.*, the peak of the strychnine convulsion.

Strychnine thresholds were determined on two control groups of mice in which the animals weighed 15-17 g, and 18-20 g respectively (Table III).

Results. The results obtained with 3 anticonvulsant drugs (sodium phenobarbital, tridione, and phenurone) and myanesin illustrate the use of the timed intravenous infusion method. These drugs were injected intraperitoneally at dosage levels which produced slight central nervous system depression. The mice were tested at the peak of drug action (usually 30 to 120 minutes). Degree of threshold elevation (Table I) is expressed as a ratio of the mean of the thresholds of the protected animals to the mean of the thresholds of the appropriate group of control animals of similar weight.

Tridione (500 mg/kg) effectively elevates the metrazol threshold. However, it does not prevent death or modify the "persistent convulsions." Both phenobarbital (50 mg/kg) and phenurone (400 mg/kg) elevate the thresholds significantly. Phenobarbital modifies the "persistent convulsions" into clonic fits but fails to lower mortality. Phenurone modifies the "persistent convulsions" and prevents fatality in 70% of the animals. Myanesin raises the thresholds only slightly when metrazol is infused, but the lethal outcome is delayed.

For purposes of comparison, the results obtained from the application of the subcutaneous metrazol method using tridione at the same dose is presented (Table II). Control experiments were performed and it was found that 85 mg/kg of metrazol produced convulsions in 95% of the mice, severe seizures in 60%, and death in 20%. Metrazol, at a dose of 125 mg/kg subcutaneously, produced severe convulsions and death in all animals tested.

The greatest advantage of the timed intravenous infusion metrazol method is that it makes possible an accurate graded evaluation of anticonvulsant compounds requiring a minimum of time and effort. Since the concentration of metrazol within the animal's blood stream is increased at a constant rate during each *single* trial, a complete picture of the antagonistic properties of a drug may be obtained from *one* series of animals using increments in dosage of 12.5 mg/kg. The subcutaneous method, however, necessitates a

TABLE III.
Intravenous Strychnine Infusion—Mice.

Groups	No. mice	Convulsion, cc	Mortality, %	Time of death
Normals, 15-17 g	60	0.19 ± .044 (1.00)	100	Immediate
" 18-20 g	20	0.21 ± .028 (1.00)	100	"
Tridione, 500 mg/kg 1 hr	40	0.28 ± .052 (1.48)	82.5	20 min.
Phenurone, 400 mg/kg 1½ hr	20	0.28 ± .042 (1.50)	60	28 min.
Phenobarbital, 50 mg/kg 2 hr	20	0.28 ± .033 (1.50)	65	19 min.
Myanesin, 300 mg/kg 10 min.	20	0.36 ± .052 (1.70)	30	42 min.

number of series, each at a different concentration of metrazol; thus, it calls for the use of many more animals and much more time, especially with effective drugs. For example: To test the efficacy of tridione (500 mg/kg) by the timed intravenous infusion method, one group of 20 animals was used. The total procedure (including weighing and injecting the animals) required 3½ hours. Employing the subcutaneous method to test tridione (500 mg/kg), and increasing the dose of metrazol in 25 mg/kg steps for each group of mice, 50 mice were used, 10 at each dosage level. The total procedure required 15 hours when each group was tested separately. Eighty mice were used to determine control responses, LD-50 and CD-50 for statistical analysis of the data for both groups.

The timed intravenous infusion method is as accurate as the subcutaneous method. Table II shows that the protection index for tridione (Ratio: tridione/control) is approximately the same by both methods of testing.

The sensitivity of the intravenous infusion method has been demonstrated frequently in the testing of new anticonvulsant chemicals. With one drug a 30% increase in dosage caused a 36% elevation of the protection index. In another instance a 20% increase in dosage caused the ratio to increase almost 200%.

The Timed Intravenous Infusion of Strychnine. The results obtained from tests made on phenobarbital, tridione, and phenurone are presented here to illustrate the validity of strychnine infusion as a standard laboratory procedure for screening anticonvulsant compounds (Table III). All 3 compounds are effective in elevating the strychnine threshold, phenurone and phenobarbital being slightly more antagonistic than tridione. 82.5% of the mice die in the tridione tests, while phenurone and phenobarbital show greater ability to protect the animals from death. Myanesin is the most effective of all since it affords protection from death in 70% of the animals and produces the greatest rise in thresholds.

Summary. The timed intravenous infusion metrazol test makes possible an accurate, economical, and rapid evaluation of anticonvulsant compounds. It presents distinct advantages over the subcutaneous method because: (1) fewer animals are needed in order to draw a statistically significant conclusion on the drug being evaluated; (2) less time is necessary to complete the testing procedure; and (3) it is equal in accuracy to the subcutaneous method.

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Experimental Toxoplasmosis.

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Investigations by others have already shown that certain of the sulfonamides[1,2] have an inhibiting effect on the growth of toxoplasma in the mouse and rabbit, but that penicillin,[3] streptomycin,[4] and certain antiprotozoal[4,5] drugs have no apparent effect. Recently, it has been shown[6,7] that when toxoplasma are inoculated into the 10-day-old chick embryo, death of the embryo regularly occurs 5 to 7 days after inoculation. We have used chick embryos infected in such a manner to determine the therapeutic effectiveness of various drugs on the disease by comparing the survival time of control embryos with infected embryos which had been treated. Additional observations on the infected mouse, rabbit and guinea pig are included in this report.

Methods and Materials. The "R.H." strain of toxoplasma isolated by Sabin[8] from a fatal human case of encephalitis was used in these studies. It was maintained in this laboratory by continuous intraperitoneal or intracerebral passage in mice. Fertile hens' eggs incubated both before and after inoculation at 37°C were inoculated with 0.2 cc of a 10% suspension of either mouse brain or peritoneal exudate into the yolk sac, using a No. 22 needle 1 inch long on a tuberculin syringe. In all instances, the initial inoculation was made in the 10-day-old fertile egg and the drug to be tested was injected in a similar manner 24 hours later. Drugs used were prepared so that the amount to be evaluated was contained in 0.2 cc, the amount injected. The following controls were run with each drug tested: drug control, toxoplasma control, and saline control. All drugs used were from commercial lot preparations and were diluted with sterile saline solution to the proper dosage just before use. After inoculation each egg was candled daily until death or hatching occurred. Chicks that hatched were sacrificed at various ages and 10% suspensions of their livers and brains in physiological saline solution were inoculated into mice to determine the presence or absence of toxoplasma. In the mice, guinea pig and rabbit studies, depending upon the instance, the organisms were either given by mouth or injected subcutaneously, or intraperitoneally.

Results. A. Drug Studies. Sulfathiazol, sulfadiazine, sudfapyridine, sulfamerazine, and sulfathiazol-sulfamerazine (combined) in varying dosages prolonged the survival of the toxoplasma infected chick embryo as shown in Table. I. Penicillin, streptomycin, aureomycin,† and acetarsone had no effect on the survival of the infected chick embryo. In the infected embryos (treated with one of the sulfonamides) that survived and were allowed to hatch, toxoplasma were recovered from the livers by mouse inoculation, in every instance regardless of the age of the chick. In the sulfonamide treated embryos, dosages used below 5 mg per 0.2 cc afforded little or no protection against the toxoplasma; dosages above 20 mg per 0.2 cc appeared too toxic to the embryo to determine its effectiveness.

* National Research Council Fellow in Pediatrics.

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† Aureomycin was kindly supplied by the Lederle Laboratories.

TABLE I.
Percentage of Survival of Infected 10-day Chick Embryos with Various Drugs.

No. of eggs	Therapy	Dose	% survival at 21 days*
96	Saline		0
12	Sulfathiazole	.1 mg	0
12	"	1 "	0
12	"	5 "	77
12	"	10 "	71
12	"	15 "	88
10	Sufladiazine	5 "	100
10	"	10 "	88
10	"	15 "	70
10	"	20 "	85
10	Sulfamerazine	5 "	66
10	"	10 "	75
10	"	20 "	77
10	Sulfapyridine	1 "	0
10	"	10 "	33
10	"	15 "	50
10	Acetarsone	.25 "	0
10	"	1 "	0
10	"	10 "	0
16	Penicillin	10 units	0
16	"	100 "	0
16	"	1,000 "	0
6	Streptomycin	10 "	0
16	"	100 "	0
16	"	1,000 "	0
16	"	10,000 "	0
20	Aureomycin	10 "	0
20	"	100 "	0
20	"	1,000 "	0
10	Sulfathiazole } Sulfamerazine }	6 mg of each	100
10	Sulfathiazole } Sulfamerazine }	12 mg of each	71

* Based on number of embryos living after subtracting inoculation deaths.

B. Congenital Toxoplasmosis in the Guinea Pig. Two gravid guinea pigs approximately one week from delivery were inoculated subcutaneously with 0.2 cc of 1:1,000 dilution of toxoplasma-containing peritoneal exudate. Five days later both guinea pigs delivered 2 living and one stillborn offspring. One of the stillborns appeared to have bilateral cataracts; sections made of most of their tissues for microscopic examination revealed no obvious toxoplasma. The living young were allowed to remain with their mothers until the mothers died about 10 days following the injection of toxoplasma. The offspring were sacrificed at varying periods up to 12 days of age in each instance toxoplasma were either found on stained imprints of body tissues or were recovered by means of inoculation into mice.

A third gravid guinea pig was infected one week before term in the same manner as described above, but before spontaneous labor ensued she was given ether anesthesia and 3 live young were delivered by Cesarian section to prevent the possibility of contamination via the birth canal. Toxoplasma were recovered from the mother's blood at delivery and were demonstrated in imprints of tissues in two of the offspring that died 24 hours later. Toxoplasma also were recovered from the brain of the third offspring that died at the age of 7 days from convulsions. However, we were unable to identify the organisms in sections of tissue from which they were isolated, even after a prolonged search.

C. Recovery of Toxoplasma from the Urine of Rabbits and Guinea Pigs. Two rabbits were inoculated intracutaneously with mixtures of toxoplasma and serum for the routine neutralization test. Two guinea pigs were inoculated subcutaneously with only a suspension of toxoplasma. After 8 days, un-

der ether anesthesia the abdominal walls were opened sterilely and the bladders were aspirated with needle and syringe. In each instance the urine was very concentrated and so was diluted 3-fold with saline solution and then inoculated intraperitoneally into mice. Approximately 7 days later all of the mice became ill with ascites and toxoplasma were found in large numbers.

On 3 occasions mice were likewise infected with toxoplasma, both intracerebrally and subcutaneously, but toxoplasma were not recovered from the urine which was removed sterilely and inoculated into other mice.

D. Failure to Infect Mice with Toxoplasma Given Orally. On 5 occasions 7 mice were fed water containing millions of fresh toxoplasma in suspension, most of which was consumed 24 hours after preparation. None of these mice developed toxoplasmosis during the one month period of observation. Mice were likewise infected with toxoplasma intracerebrally and intraperitoneally and placed in cages with healthy litter mates to ascertain if infection could be acquired by cannibalism. In all instances the infected mice died 4-5 days after inoculation and were consumed by the normal litter mates. Under such circumstances, none of these litter mates developed toxoplasmosis.

E. Failure to Recover Toxoplasma from the Infected-Immune Rabbit. Three rabbits that had been used for the rabbit neutralization test but had recovered from their infection and had a high titer of complement fixing antibodies in their sera were sacrificed. The rabbits were selected so that one had recovered from its infection 1 month, one but 3 months, and one a considerably longer time, namely 19 months. In each instance sections of the brain, liver, and spleen were removed sterilely from the rabbit and made into a 10% suspension with saline. These suspensions (0.5 cc) were inoculated intraperitoneally into each of 3 mice who failed to develop toxoplasmosis after one month of observation.

Discussion. The results of the chick embryo method for testing the effectiveness of various drugs on toxoplasmosis compares favorably with previous studies done in intact animals.[1-5] This technique is relatively simple and non-time-consuming. Although it was thought that acetarsone produced some clinical improvement in acute toxoplasmosis in certain patients,[9] tests in the chick embryo showed no effect of the drug on the organisms. Aureomycin is known to be effective against experimental brucella infection in the chick embryo,[11] however, we were unable to show any inhibiting effect of the drug on the growth of toxoplasma.

While congenital toxoplasmosis was produced in the guinea pig, as proven by isolation of the organism from the brain, examination of the tissue sections microscopically revealed no organisms. This confirms our impression of the difficulty in making a diagnosis from pathologic specimens without the aid of biologic tests.[9]

Since we were unable to infect mice orally with toxoplasma, transmission from animal to animal does not appear to occur via the gastrointestinal tract. The significance of the presence of toxoplasma in the urine of infected rabbits and guinea pigs is not clear at the present time. Apparently, the infected rabbit, as well as the monkey[10] in certain instances, can recover completely from the infection.

Summary. The effectiveness of certain of the sulfonamides and the ineffectiveness of certain antibiotics in suppressing toxoplasmosis in the chick embryo has been demonstrated. Congenital toxoplasmosis was produced in the guinea pig.

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16893

Histamine in Rat Plasma; Correlation with Blood Pressure Changes Following X-irradiation.

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Although considerable has been written to indicate that there is a close correlation between the conditions of shock and the liberation of a histamine like substance from damaged tissue[1,2,3] it is only recently that the publications of Prosser, Painter and Moore[4] and others[5,6] suggest that a similar histamine shock syndrome can consistently be produced with mid-lethal dosages of X-rays. Their observations indicate that with such concentration of X-rays the toxic products of tissue breakdown reach their highest concentration at about 2 hours after irradiation.

In view of the fact that no definite statement is available as to whether the toxic product liberated is actually histamine or not, it will be the object of this investigation to demonstrate that the toxic product in the blood stream following irradiation is histamine, and that the level of assayable histamine in blood is highest about 2 hours after irradiation and again about 5 days later, at which times the blood pressure of the animals is at its lowest point.

Experimental. In the experiments to be reported, adult rats were used irrespective of sex. After irradiation, the rats were used either for the collection of blood samples (enough for 2 cc of plasma) to be assayed for histamine at various time intervals, or for blood pressure determinations over the corresponding time interval. In making these determinations a total of 21 rats were used for making histamine assays at various time intervals, varying from .5 to 24 hours after irradiation; 5 other rats were used to follow variations in blood pressure, determined at 15 minute intervals for 4 to 5 hours after the radiation was given. After once being irradiated, never was a rat used for a second experiment.

The irradiation techniques used were developed by Quastler and Kirschner.[3] The rats were placed in a cell cut out of presswood, large enough to accommodate 3 rats. The factors for the raying were: 200 KVp without a filter. The distance from the target electrode to the top of the rats' cell was 50 cm. One-half of the dose was administered from the top and one-half from the bottom. By raying in this fashion a fairly homogeneous distribution of dose is attained. The dose rate was 100 r per 2 minutes and 24 seconds, and the actual mid-lethal dose given each rat was 600 r.

When blood pressure measurements were taken on the intact rat to record the severity of the effects of irradiation, a slight modification of the method described by Byrom and Wilson[7] was used. It consisted essentially in using a plethysmograph especially designed to fit the rat's tail.

The method used for preparing extracts for histamine assay was that developed by McIntire, Roth and Shaw.[8] This method appears to be superior to others available[9,10,11]

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7 Byrom, F. B., and Wilson, C., *J. Physiol.*, 1938, **93**, 301.

8 McIntire, F. C., Roth, L. W., and Shaw, J. L., *J. Biol. Chem.*, 1947, **170**, 537.

9 Code, C. F., *J. Physiol.*, 1937, **89**, 257.

10 Barsoum, G. S., and Gaddum, J. H., *J. Physiol.*, 1935, **85**, 1.

11 Schild, H. O., *J. Physiol.*, 1942, **101**, 115.

in that very small amounts of histamine can be detected and also that only histamine is said to be extracted from the blood sample being studied and not other impurities. The essential features of the method are that the histamine can be removed from the aqueous sample almost in its entirety with N-butanol, and that then the histamine can be eluted from the butanol by means of the cation exchange medium, cotton acid succinate. Following this the histamine is washed from the

TABLE I.
Amount of Histamine Present in 2 cc Samples of Blood Plasma After Irradiation (600r).

Rat No.	Sampling time after x-ray (hr)	Histamine present (γ)
A4	.5	.0
A13	.5	.0
B1	1.5	1.0
A2	2.	1.0
A3	2.	2.0
A5	2.	2.0
A15	2.	1.2
A20	2.	2.0
A22	2.	1.4
B2	2.	1.2
B3	2.	1.2
C1	2.	1.6
C2	2.	1.6
B4	2.5	.4
A21	3.	.0
A23	3.	.0
A24	4.	.0
C3	10.	.0
C4	16.	.0
A25	18.	.0
A26	24.	.0

cotton acid succinate with a small volume of dilute hydrochloric acid, and the resulting solution is neutralized with weak sodium hydroxide to give an isotonic solution suitable for bioassay. The actual assay for histamine is carried out by recording blood pressure changes in the anesthetized cat (Nembutal).

Results. In reporting the results obtained, particular emphasis will be placed on the correlation between the appearance of assayable histamine in the blood plasma and the maximum fall in blood pressure occurring approximately 2 hours after the mid-lethal dose of radiation was given (600 r). The data obtained for the actual amount of histamine present in terms of gamma per 2 cc of blood plasma is presented in Table I. That the assayable compound in these experiments is histamine is supported by the fact that the method used for isolating the compound is specific for histamine. It was also found that if a known amount of assayable histamine in gamma concentration which was previously extracted from a blood sample of an irradiated rat, is added to a similar amount of histamine diphosphate, the fall in blood pressure in the test cat was consistently twice that which

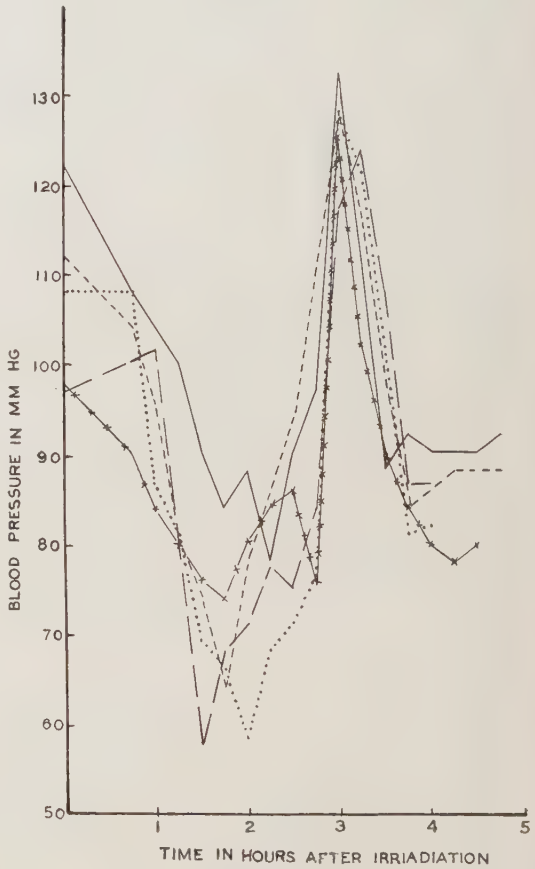

FIG. 1.
Effects of irradiation on blood pressure in the rat.

occurs when the histamine diphosphate is injected alone. It is believed that if the extractive is not actually histamine it should not produce so clear cut an additive effect as these experiments indicated.

It was interesting to note that along with the appearance of histamine there occurred a marked fall in blood pressure which showed an excellent correlation between the highest

level of histamine concentration in the blood and the lowest point of blood pressure fall (Fig. 1). As the graph indicates, following this fall in blood pressure there occurs a marked compensation within an hour that exceeds the normal pressure at the beginning of the experiment. Although there is no satisfactory explanation for this change in blood pressure after the marked fall, it should be mentioned that when this rise does occur there is no measurable histamine present in the blood stream.

In a limited number of experiments in which the rats were followed for a longer time after irradiation than those just described, it was interesting to find that following the first 4 or 5 hours period after mid-lethal dosage of X-rays, even though the histamine in the blood was not measurable, the animals' blood pressure remained relatively low compared with normal, and continued to fall slowly to levels of 60 to 70 mm of Hg by the 4th to 6th day after irradiation. There also occurred in all the animals studied a marked loss in appetite and weight during this same period.

From this critical point, however, the rats either became progressively more depressed and died at the end of about 10 days, or showed a marked recovery in appetite, weight, blood pressure, and general appearance and survived the 600 units of X-irradiation. It was also interesting to note that if histamine assays were run on rats in these experiments there occurred a gradual increase in assayable histamine starting the 2nd day after irradiation, until on the 5th day, the rats showed nearly 1.0 γ of histamine (by assay) per 2 cc of blood plasma extracted. If the rats survived this critical 4 to 6 day period and showed signs of recovery, there was no evidence of histamine present in the blood by the 9th or 10th day after irradiation.

From these experiments it would appear that the low blood pressure correlates with high levels of histamine in the blood plasma which is very suggestive of the theory that the cause of death after irradiation is of the nature of circulatory failure or a condition simulating shock, an observation that was earlier suggested by Prosser, Painter and Moore.[4] Although no explanation can be advanced concerning the mechanism responsible for the appearance of histamine after irradiation, it is believed that the cyclic appearance of histamine at a 2 hour and a 5 day period after irradiation offers suggestions for further investigation of the problem in question.

Summary. 1. Rats, after X-irradiation with mid-lethal dosage (600 r) show assayable histamine in concentrations of 1 to 2 γ per 2 cc of blood plasma at 2 hour and 5 day intervals following the irradiation.

2. Rats irradiated similarly to those used in the histamine assay experiments show a fall in blood pressure that correlates with the appearance of histamine in blood plasma and strongly suggests that histamine is responsible for the lowered blood pressure.

3. Rats, from 4 to 6 days after a mid-lethal dosage of irradiation show a loss in appetite and weight, and a critical fall in blood pressure from which only 50% will recover. The rest die within 9 or 10 days after the irradiation was given.

16894

Chorio-meningo-encephalitis Following Inoculation of Newcastle Disease Virus in Rhesus Monkeys.*

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Newcastle Disease Virus (NDV), commonly the cause of a disease of fowl, patently, may, because of intimate association with man, cause human disease. Recognized syndromes in man caused by NDV include conjuncti-

* Aided by a grant from the United States Public Health Service.

vitis,[1] and an influenza-like illness.[2] Recently, Howitt, Bishop, and Kissling[2] have pointed out that NDV may cause a mild CNS disease indistinguishable from abortive poliomyelitis in infants and children. In view of this last mentioned observation a study was made and Newcastle Disease Virus has been found to cause a CNS disease in rhesus monkeys. The results of this study are reported herein.

Materials and methods. Virus. The "Manhattan" strain of NDV† was grown in 10 day old embryonated eggs. A pool of infected allantoic fluid (12th passage) was made and stored at −70°C. The infected fluid had an embryo MLD in dilution 0.1 cc $\times$ $10^{-8.5}$. The hemagglutinin titer (fowl RBC) was 1:640. The hemagglutinin and hemagglutinin-inhibition tests were done with modifications as described by Florman.[3]

Monkeys. Rhesus (*Macaca mulatta*) monkeys were used. Each monkey received 0.8 cc of infected, undiluted allantoic fluid in the brain. Monkeys were observed twice daily. Temperature records were kept. Whole blood, serum and cerebrospinal fluid were obtained before and at intervals following inoculation. The CNS obtained from monkeys was examined histologically; fresh material was passaged to monkeys, rodents and embryonated eggs.

Results. Five monkeys were inoculated with infected allantoic fluid. All became sick. One died on the 6th day (following inoculation): one other was sacrificed on the 7th day. The remaining 3 monkeys recovered.

Clinical course. The clinical course of one monkey appears in Fig. 1. Following an incubation period of 2 to 5 days fever (104 to 105°F) occurred. With the onset of fever monkeys became agitated, and tremulous. They were grossly awkward in locomotion. The fever lasted 2 to 7 days. Convalescence was slow; symptoms lasted 7 to 10 days following abatement of fever. Cerebrospinal fluid obtained from 3 monkeys in the acute phase of illness was cloudy. The cell count ranged from 37 to 250/mm³. Mononuclear cells predominated.

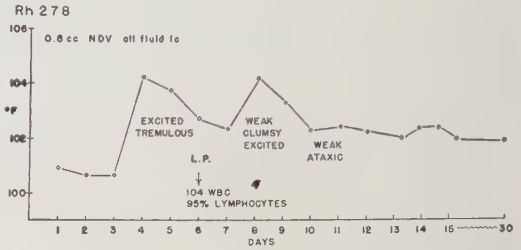


FIG. 1.

Response of a rhesus monkey following intracerebral inoculation of Newcastle Disease virus.

Serologic Studies. Hemagglutinin-inhibition tests with pre-inoculation, acute and convalescent serums showed an 8 to 32 fold rise in NDV antibody level. Specific complement-fixation was obtained with NDV infected, allantoic fluid. No complement-fixation was obtained with lymphocytic choriomeningitis infected mouse brain.[6]

Passage. The CNS of a rhesus monkey sacrificed during the acute phase of illness was inoculated intracerebrally into 2 rhesus monkeys, Swiss mice, hamsters, guinea pigs, cotton rats and embryonated eggs. Chick embryos died; NDV was recovered from them. A monkey sickened. The sick monkey was unable to sit up on the 30th day. There was ipsilateral weakness of the right arm and leg. All other animals remained well (31 days).

Pathology. The CNS histology was characterized by (a) encephalitis, (b) focal meningitis, and (2) inconstant and intense inflam-

[1] Burnet, F. M., *Med. J. Australia*, 1943, **2**, 313.

[2] Howitt, B. F., Bishop, L. K., and Kissling, R. E., *Am. J. Pub. Health*, 1948, **38**, 1263.

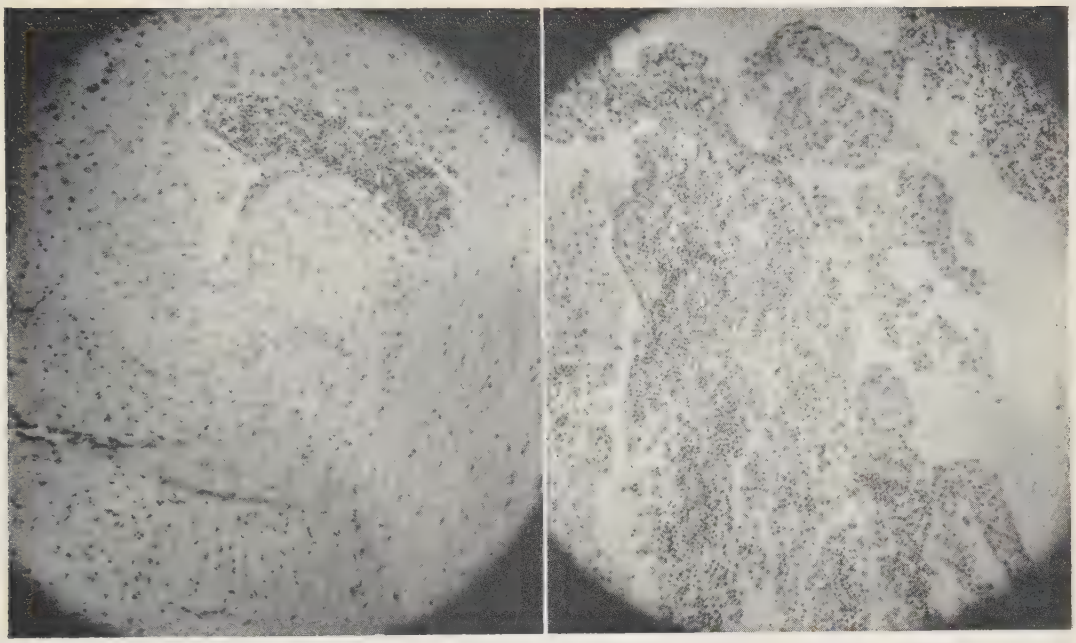
† We are indebted to Dr. L. D. Bushnell, Department of Bacteriology, Kansas State College, Manhattan, Kansas, for the "Manhattan" strain of Newcastle Disease Virus. On its receipt, the virus was in the 8th passage in the embryonated egg. Infected allantoic fluid (12th passage) inoculated intracerebrally in Swiss mice, guinea pigs, hamsters, and cotton rats, caused illness in cotton rats[4] and hamsters.[5]

[3] Florman, A. L., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 548.

[4] Wenner, H. A., unpublished observations.

[5] Reagan, R. L., Lillie, N. E., Hauser, J. E., and Brueckner, A. L., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 293.

[6] Espana, C., and Hammon, W. McD., *J. Immunol.*, 1948, **59**, 31.



(1) (2)

FIG. 2.

Lesions in the (1) rhombencephalon and (2) choroid plexus of a rhesus monkey following intracerebral inoculation with Newcastle Disease virus.

mation of choroid plexus (Fig. 2). Perivascular cuffing, neuronecrosis and neuronophagia were best seen in the rhombencephalon, particularly in juxtaposition to the 4th ventricle. No changes were seen in the spinal cord of the monkeys inoculated with NDV infected allantoic fluid. In the passage monkey additional pathologic changes were found. These changes consisted of extensive inflammatory and degenerative changes in the gray matter, particularly in the anterior horns of the spinal cord.

Discussion. Heretofore, fowl and hamsters have been found susceptible to Newcastle Disease Virus. Now another host, the *rhesus* monkey has been found susceptible to NDV.‡

‡ This virus causes encephalitis in cotton rats also.[4]

The pathogenesis of NDV in rhesus monkeys requires additional clarification. At the height of illness NDV can be detected in CNS tissue and occasionally in the blood, but not in cerebrospinal fluid. If the pathological lesions only are considered it would appear that NDV may have been disseminated in cerebrospinal fluid. (CSF) The apparent absence of virus in CSF, in contrast to the presence of it in blood and CNS tissue may indicate widespread proliferation of virus. Further study is necessary in order to determine whether NDV resembles in pathogenesis experimental lymphocytic choriomeningitis or possibly poliomyelitis.

Summary. The "Manhattan" Strain of Newcastle Disease Virus produced in rhesus monkeys a chorio-meningo-encephalitis.

16895

Effect of Subcutaneous Injury on Tumor Growth in the Mouse.*

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Several devices were explored for inducing inflammation under the skin of the mouse, to be followed by tumor implantation. Among these were the injection of acid, alkaline, or distilled water; the implantation of a small wad of glass wool; the injection of an air bleb, alcohol, turpentine, or histamine; and mechanical separation of the skin from the abdominal muscle wall. None but the last mentioned of these irritants produced consistent alterations in the growth of implanted tumor fragments. The type of inflammation induced by mechanical injury was, therefore, studied in detail in respect to its effect on tumor growth, and this effect comprises the main subject of this paper.

Procedure. A sharp-pointed 3-inch surgical scissors was inserted under the skin in the inguino-abdominal region and directed anteriad to a point about one-fourth inch from the axilla. With the position of the scissors' fulcrum lying at the point of original entry, the scissors was opened broadly so that the outer surfaces of its shears radically cleaved the skin from the underlying muscle tissue, without however puncturing the skin. Local bacterial infection was not evident in mice so treated, even when rigid surgical asepsis was not observed.

Groups of mice were so prepared for tumor implantation. Into some of these mice, fragments (approx. 2 mm^2) of sarcoma 180 were implanted immediately after the cleavage operation by inserting a trocar through the incision made by the scissors; the seeding fragment was deposited as close as possible to the center of the cleaved area. Fragments of comparable size and from the same donor tumor were implanted in the corresponding region of mice which had not been given the cleavage treatment; these served as controls.

Additional experiments were set up in which groups of cleavage-treated mice received tumor implants at various intervals after cleavage: at 24 hours after cleavage, at 48 hours, 72 hours, 6 days, and 12 days. In each instance tumors were implanted into parallel control groups of non-cleaved animals. The implanted fragment was allowed to grow for the usual 6 to 7 day period, at the end of which the tumorous growth was dissected out and wet-weighed. Before weights were taken, a puncture was made of the dissected tissue mass and exudative fluid was drained off. Rockland white male mice of 18-22 g were used throughout.

Results. For the first 4 days following fragment implantation into the cleaved subcutaneous area, tumor growth appeared to progress normally and was indistinguishable topographically from that of the controls. Between the 4th and 6th day after implantation a very marked swelling would become apparent in the area where the implant was deposited. Within 24 to 48 hours after the onset of this swelling, the experimental tumor appeared to attain a mass of from 5 to 10 times that of the control tumor (Plate I, Fig. 1 & 2). Upon dissection it was found that an extensive vesicle had developed throughout the cleavage area; and that a bleb of exudate had formed in close association with the growing tumor fragment (Plate I, Fig. 3). The vesicle fluid accounted for about half the total volume of the swelling. By the 9th day a hardening of the soft mass became evident. By the 12th day the edematous fluid was considerably replaced by the ingrowing tissue; the mouse in all such cases died before this process was complete, death usually occurring between the 12th and 15th day after implantation.

Animals implanted with tumor fragments immediately after cleavage exhibited the

* The work reported in this paper is a portion of that being carried on under a grant from the National Advisory Cancer Council of the U. S. Public Health Service.

TABLE I.

Weights of tumors (sarcoma 180) dissected from mice during the 6-7 day period after implantation. Control mice had received no previous treatment; others were subjected to skin-muscle cleavage at various intervals before implantation (see text).

		Interval between cleavage operation and time of tumor fragment implantation			
Exp. No.	Control g	0-hr g	24-hr g	48-hr g	72-hr g
1	.15	.72	.47		.58
	.28	.39	.21		.42
	.15	.59	.47		.27
	.30	.22	.73		.12
	.20	.63	.12		.41
	.06				
	.25				
2	.20			1.00	
	.18			.45	
	.33			.54	
	.33			.41	
	.24			.67	
				.66	
				.16	
3	.25	.77	.48		.32
	.18	.89	.53		.47
	.18	.55	.63		.54
	.33	.47	.13		.24
	.20	.47	.86		.41
	.42	.19			.44
	.23				.20
	.18				
4	.46	.69	.46	.62	
	.51	.45	.31	.84	
	.26	.36	.33	.39	
	.29	.25	.81	.51	
	.27	.45	1.17	.61	
	.31	.71	.70	.70	
		.45	.18	.21	
		.87			
5	.39	1.10			
	.26	.86			
	.24	.31			
	.20	.96			
	.16	.28			
	.22	.74			
		.90			
Mean tumor wt	.25	.59	.50	.54	.36

above-described effect to an extent not distinguishable from that in animals in which the implantation was made 24 or 48 hours after cleavage. The effect showed signs of lessening in animals implanted 72 hours after cleavage; was barely apparent in those implanted 6 days after cleavage; and was completely absent in animals implanted 12 days after cleavage, at which time, apparently, complete healing of the original injury had occurred.

The data of Table I indicate that the mean weight of tumor tissue from cleaved animals sacrificed 6 to 7 days after implantation was in the order of twice that of the non-cleaved controls. It was noted, on the other hand, that had weighing been done at a later date, say during the 8-10 day period, the difference between cleaved and control groups would probably have been recorded as considerably greater. In Table I it will be noted, too, that the growth-enhancement effect occurred in

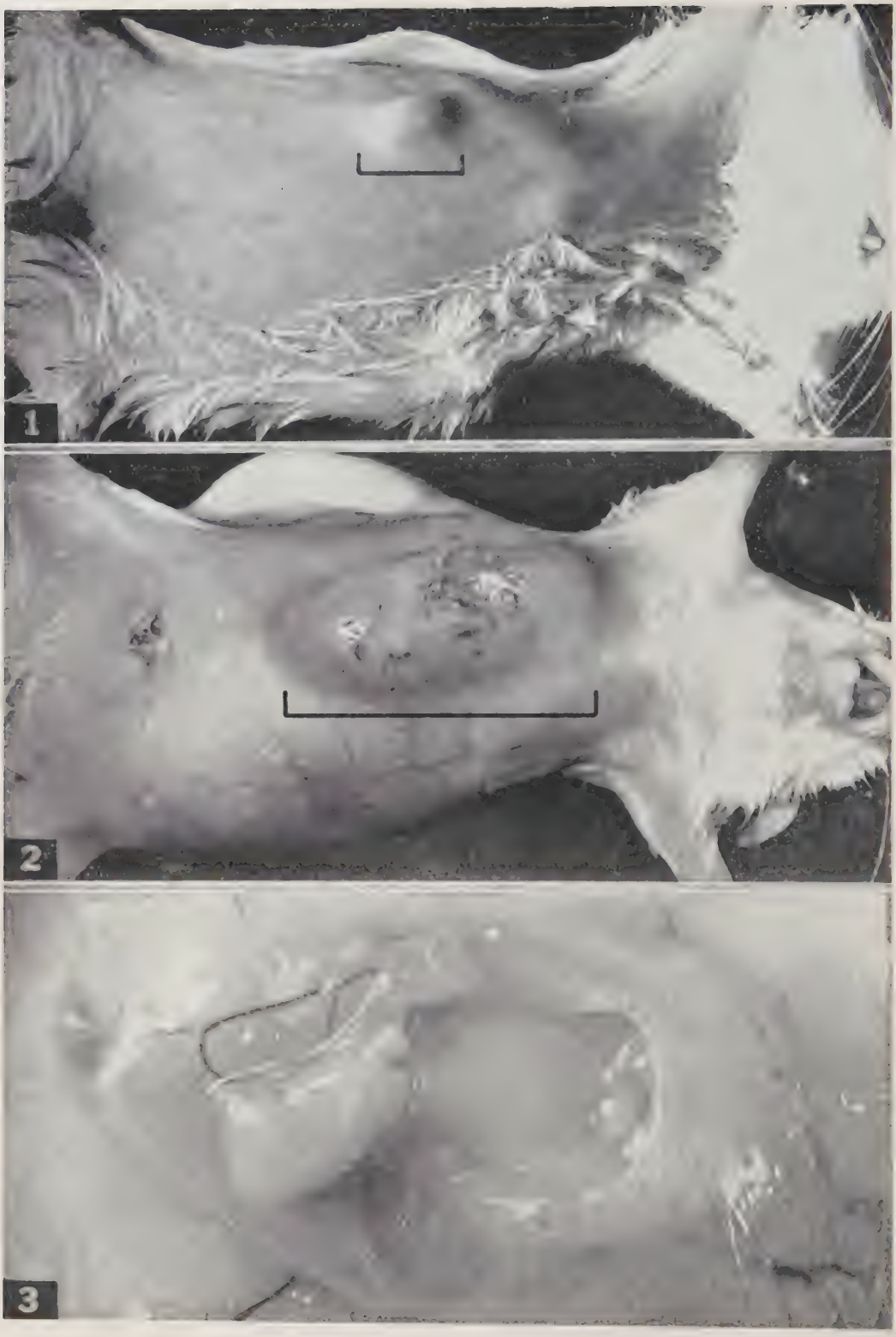
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PLATE I.
Abdominal aspect of epilated mice to show relative growth of sarcoma 180 implant.
FIG. 1. Control tumor at 6-7 day stage (approx. ×2).
FIG. 2. Cleavage tumor at 6-7 day stage (approx. ×2).
FIG. 3. Cleavage tumor with skin removed and edematous bleb cut open (approx. ×4).

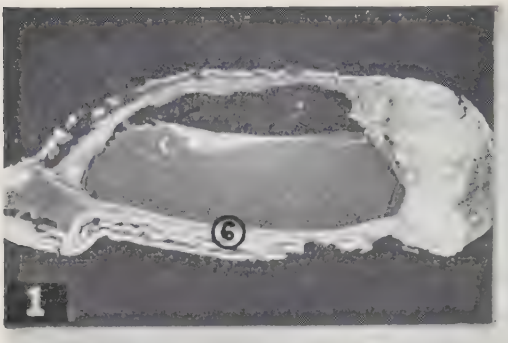
about 80% of the cleavage-treated mice, the remainder showing normal tumor growth. Occasionally an animal would lose its newly implanted fragment; such animals were rejected from the experiment.

Exudate withdrawn by syringe from the edematous bleb 6 days after tumor implantation was a pale pink serum, of pH 7.6, which when examined fresh or as a Wright's stain smear showed a sparse distribution of erythrocytes, some cells resembling lymphocytes and monocytes, and some polymorphonuclear leucocytes—a picture confirmed in Bouin-fixed hematoxylin-and-eosin sections of the tissue and bleb mass. At the interface of the serum and its encasing tissue (which appeared to be an intermixture of proliferating sarcoma cells and connective tissue elements) loose strands and masses of tumor cells extended inward, containing many cells in active mitosis (Plate II, Fig. 3, 4, & 5). The occurrence of such an inward infiltration was confirmed by examination of tissue fixed at 6, 8, 10, and 12 days after implantation. Very clearly, the induration of the mass palpably observed during the 8-12 day period was due to this ingrowth. Interspersed among the tumor cells at the growing fringe at the aforementioned interface were polymorphs, although their number was not such as to suggest suppuration, except in later stages when necrosis in the center of the vesicle often became more marked. Concomitant with tumor growth and the thickening of the vesicle wall, the primary tumor mass became undemarcably involved.

In further experiments a number of mice were subjected to the cleavage operation, but were not implanted with tumor tissue. Mice so prepared were killed at 1-day intervals following cleavage; the abdominal skin was incised and retracted so that observation could be made of the gross damage resulting from the cleavage. In most cases the inner surface of the cleaved skin showed some extravasation of blood, derived, obviously, from rupture of the larger vascular channels of the connective tissue during the scissors-cleavage procedure. The visible remains of such extravasation diminished after the 3rd day, and was no longer observable after the 6th. Restoration of fusion of the skin with the abdominal wall was not clearly complete until about the 5-6 day stage, as could be determined by the relative ease with which the skin could be withdrawn from the abdominal wall during dissection. Suppuration was never observed. The inner skin surface in the region cleaved showed some hyperemia, but never in conspicuous degree, and this hyperemia disappeared during the 3-6 day period. None of some 30 cleavage-control animals examined showed any discernible swelling of the area or any external evidence of edema.

The pronounced exudation observed in cleavage animals receiving tumor implants appeared to result from a specific interaction between the actively proliferating tumor tissue and the injured connective tissue substrate. Evidence of such a specificity was derived from an experiment in which fragments of muscle, tongue, intestine, spleen, and liver, instead of tumor tissue, were implanted into the cleaved area. In no instance did a palpable edema develop. The implanted non-tumorous tissue fragments were eventually resorbed without incident.

Comment. To interpret the interaction between tumor growth and the type of subcutaneous injury herein described, it may be of advantage to picture the events occurring under the mouse skin following cleavage injury. Moreover, it is necessary to consider what occurs when a tumor fragment is implanted in the usual fashion into non-injured subcutaneous tissue. In the latter case, the insertion of the trocar probably does not tear but merely distends the connective tissue. Thus the little damage that may occur is almost mechanically repaired at withdrawal of the trocar. Similarly, when the fragment is deposited, the connective tissue is stretched somewhat, but not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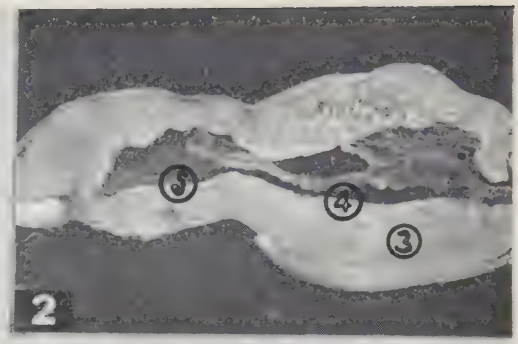
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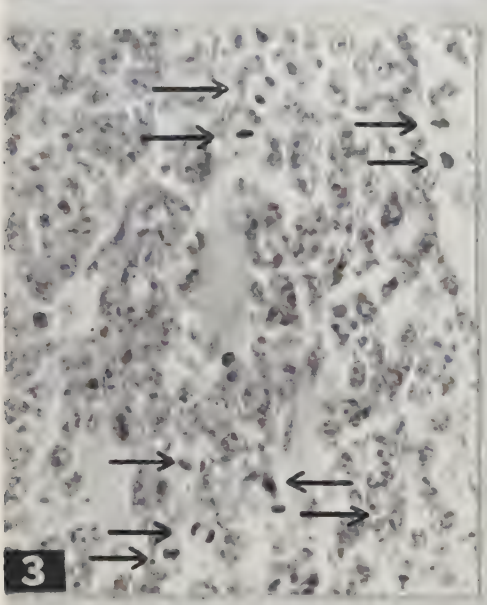
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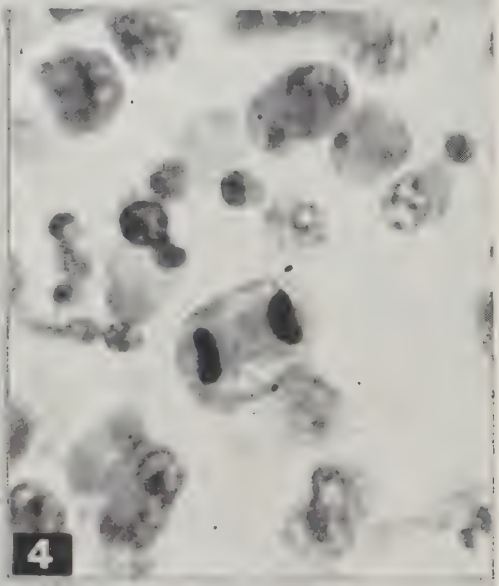
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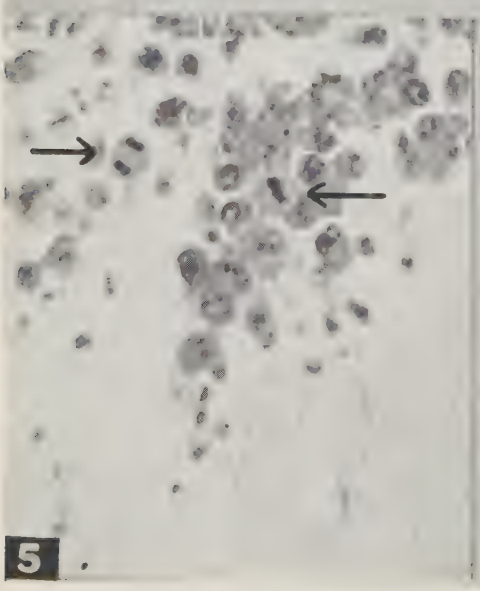
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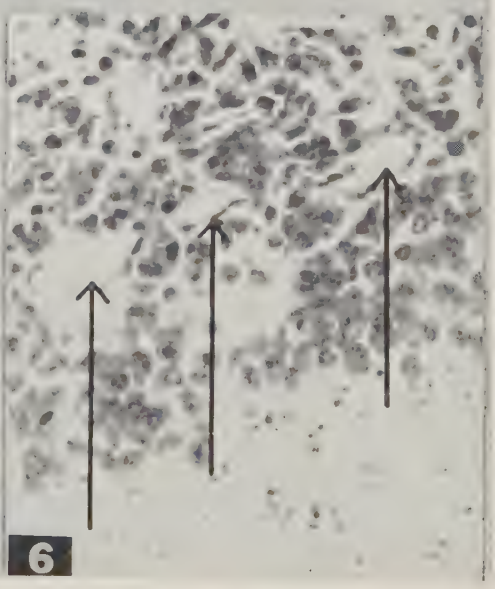
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PLATE II.

FIG. 1. Shadowgraph of median sagittal section of 6-7 day cleavage tumor (approx. ×2.5).
FIG. 2. Shadowgraph of median sagittal section of 9-10 day cleavage tumor (approx. ×2.5).
FIG. 3. Area from Fig. 2, as indicated. Note large number of mitoses (high dry).
FIG. 4. Area from Fig. 2, as indicated, to show mitotic sarcoma cell (oil immersion).
FIG. 5. Interface between vesicle fluid and growing tumor surface, from Fig. 2, as indicated. Note mitoses (high dry).
FIG. 6. Interface between vesicle fluid and growing tumor surface, from Fig. 1, as indicated. Note new capillary channels (high dry).

radically torn. During such normal implantation procedure there is little or no vascular rupture nor extravasation of blood.

The initial obvious reaction of the connective tissue to a tumor implant is one of classical imflammation, involving, hyperemia, appearance of polymorphs, and subsequently of mononuclear leucocytes.[1-3] The connective tissue area surrounding the new implant almost immediately becomes hyaline, and within a few days becomes infiltrated as a firm and expanding tumor mass. The original fragment does not change appreciably in size or shape (although its cells start undergoing degenerative changes almost at once after implantation) until about the 9th day when a fusion with surrounding necrotizing tissues renders its outlines and structure somewhat amorphous.

Following implantation of a tumor fragment, it is probable that phagocytic cells—as they do in a localized bacterial infection—attack the tumor cells at the periphery of the fragment. The tumor cells obviously win in the ensuing competition, for they proliferate vigorously and at the same time stimulate the growth of capillaries and, later, of larger vascular channels; in the meantime attracting large numbers of reticulo-endothelial elements. One of the fundamental differences between malignant and non-malignant tissue lies in the former's capacity to survive phagocytic or lytic onslaughts and to excite vascular proliferation in the substrate tissue.

Is the explosive edema described in this paper a tissue shock reaction due to the super-permeability of cleavage-inflamed capillary walls? If so, why is it elicited specifically by a tumor fragment and not at all by a normal tissue implant? It seems probable that cleavage injury, being vastly greater than that produced during simple trocar implantation, excites an excessive inflammatory and reparative response on the part of subcutaneous tissues involved. When into this dynamically unstable substrate a fragment of tumor tissue is implanted, the proliferative and stimulatory factors presumably released by the metabolizing tumor fragment find a richly vascularized and nutritious substrate in which to act or re-act.

The type or irritation produced by turpentine has been reported as not affecting carcinogenesis by benzpyrene,[4,5] which is consistent with our failure to note tumor growth enhancement by turpentine as well as by the variety of other irritating agents tested. Croton oil, on the other hand, has been reported as markedly augmenting chemical carcinogenesis, presumably by the exertion of a precarcinogenic action on skin tissues so treated.[6] Perhaps more cogent to the present discussion is the report that deep incisions made in the skin of mice previously treated by carcinogenic compounds showed localization to some extent of subsequent tumor growths.[6] It has been suggested, too, that cancer cells find a favorable environment in the region of healing wounds.[7] Whether such actions as these are related or analogous to the type of injury effect described in this paper is difficult to determine, due mainly to the variety of technics and the diversity of tumor types em-

1 Zahl, Paul A., and Drasher, M. L., *Cancer Research*, 1947, **7**, 658.

2 Zahl, Paul A., and Waters, L. L., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 304.

3 Woglom, W. H., *The Study of Experimental Cancer*, New York City, 1913.

4 Beck, S., *Brit. J. Exp. Path.*, 1938, **19**, 319.

5 Berenblum, I., *Cancer Research*, 1941, **1**, 44, 807; *Arch. Path.*, 1944, **38**, 233.

6 Pullinger, B. D., *J. Path. and Bact.*, 1943, **55**, 301.

7 Kline, B. E., and Rusch, H. P., *Cancer Research*, 1944, **4**, 762.

ployed.

Summary. When a subcutaneous inflammation is induced in the mouse through mechanical injury, and when into such a traumatized site a fragment of experimental sarcoma is deposited, a striking edematous reaction ensues, accompanied by a conspicuous acceleration of tumor growth; these associated phenomena being well defined by the 4th to 6th day after implantation.

This effect is construed as a specific interaction between the neoplastic characteristics of the tumor fragment and the cellular and/or vascular sequences of subcutaneous inflammation. The effect does not occur when nonmalignant tissues are similarly implanted.

16896

Veratrinic Effects of Pentamethylenetetrazol (Metrazol) and 2,2-Bis (P-Chlorophenyl) 1,1,1 Trichloroethane (DDT) on Mammalian Neuromuscular Function.*

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An extraordinarily diverse group of agents produces in excitable tissues a stereotyped, veratrinic effect: a single brief stimulus evokes a burst of repetitive responses.[1,2] Incomplete evidence indicates that the appearance of the veratrinic response in nerve fiber always is accompanied by a similar alteration in muscle fiber, and suggests that repetitive response is a general phenomenon which occurs in all excitable tissues exposed to veratrinic agents.

To explore further this general phenomenon, the study reported here was made of two agents which produce altered states of excitability. *Pentamethylenetetrazol* (metrazol) was selected because of its predominant effect on central neural activity and investigated for its effect on the peripheral neuromuscular unit. *2,2-bis (p-chlorophenyl) 1,1,1 trichloroethane* (DDT) was studied in the mammalian preparation because recent extensive investigations have demonstrated its potent veratrinic effect on the peripheral nerve of both Hexapoda and Crustacea.[3,4]

Methods. Details of the preparation and recording technics have been described.[2] Briefly, adult rats under pentobarbital anesthesia were used throughout. Isometric myograms were made from the triceps surae by means of resistance-wire strain gauges and recorded by means of a cathode ray oscillograph. Electromyograms were recorded through needles placed in the belly of the muscle. Electroneurograms were recorded from the sciatic-tibial nerve which had been isolated centrally and peripherally, Square-wave supramaximal stimuli were delivered to the sciatic nerve or directly to the curarised muscle. Metrazol was injected intraperitoneally (i-p) in doses of 25 to 100 mg/kg. DDT† was suspended in peanut oil-saline homogenate by means of lecithin (Asolectin)‡[5] and injected i-p in doses

* Work performed under a contract between the Office of Naval Research, U. S. Navy Department, and the Johns Hopkins University.

1 Krayer, O., and Acheson, G. H., *Physiol. Rev.*, 1946, **26**, 383.

2 Eyzaguirre, C., Folk, B. P., Zierler, K. L., and Lilienthal, J. L., Jr., *Am. J. Physiol.*, 1948, **155**, 69.

3 Roeder, K. D., and Weiant, E. A., *Science*, 1946, **103**, 304.

4 Welsh, J. H., and Gordon, H. T., *J. Cell. Comp. Physiol.*, 1947, **30**, 147.

† A twice-crystallized sample was kindly provided by the Medical Division, Army Chemical Center, Edgewood, Md.

‡ Generously provided by Associated Concentrates, Inc., Woodside, Long Island, N. Y.

of 50 to 100 mg/kg. Neostigmine methylsulfate was injected i-p in doses of 50 μg/kg.

Measurements of refractory period of nerve were made by delivering pairs of supramaximal stimuli through the same electrodes at various short intervals and determining the amplitude of the second response in relation to the first.

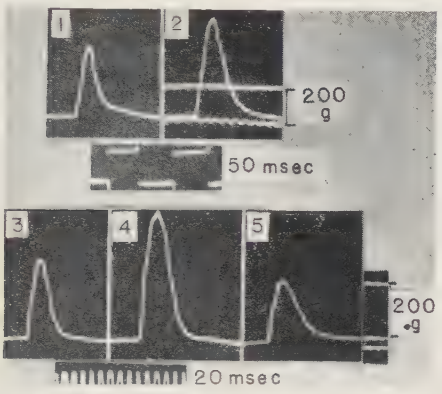

FIG. 1.
Myograms. 1. Control (indirect stimulation). 2. After 8 mg metrazol (resting tension 200 g). 3. Completely curarized muscle (direct stimulation). 4. After 20 mg DDT. 5. After 30 mg quinine diHCl (resting tension 35 g).

Results. Qualitatively, the effects of metrazol and of DDT were indistinguishable and are presented together. Both agents evoked in muscle a myotonic response which mimicked faithfully the functional and pharmacological characteristics of the myotonia occurring spontaneously in man and goat.[6] The myotonic response was seen as an increase in tension and duration of the twitch evoked by a single brief stimulus,[7] whether delivered indirectly through the sciatic nerve or directly into the curarised muscle (Fig. 1). The electrical basis of the augmented mechanical response was seen in the corresponding electromyograms, where the normal diphasic action potential was followed by a train of spikes (Fig. 2). Similar repetitive responses followed mechanical stimulation by tapping. Repeated stimulation at a rate of 12/min. produced a progressive waning of the myotonic response; the phenomenon of "warm-up". The isolated sciatic nerve studied *in situ* responded with a burst of spikes to a single stimulus following injection of either metrazol or DDT (Fig. 3).

Examples are given of the effect of agents which suppress the repetitive phenomenon: quinine (Fig. 1), Mg^{++} (Fig. 2) and Ca^{++} (Fig. 2 and 3). Accentuation of the myotonic effect by K^{+} is seen in Fig. 2.

The effect of an injection of neostigmine, which itself did not produce repetition at the neuromuscular junction, was related to the amount of metrazol which had been given. After a small dose of metrazol (25 mg/kg), which produced a few random spikes, neostig-

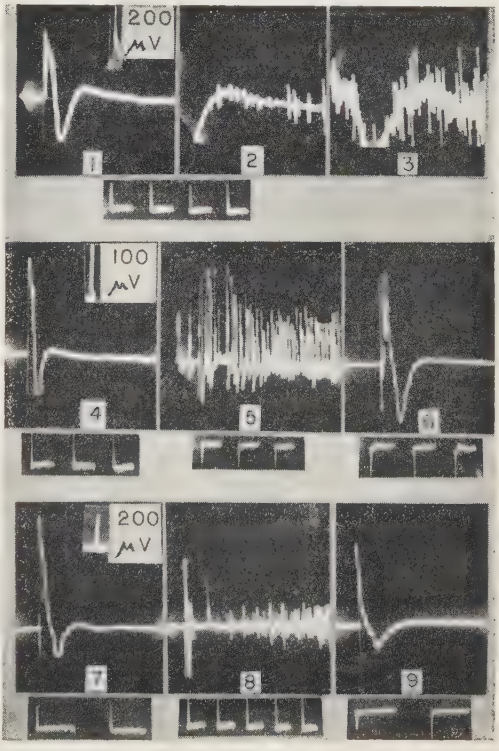

FIG. 2.
Electromyograms. 1. Control (indirect stimulation). 2. After 5 mg metrazol. 3. After 60 mg KCl. 4. Control (indirect stimulation). 5. After 20 mg DDT. 6. After 100 mg Ca gluconate. 7. Curarized control (direct stimulation). 8. After 20 mg DDT. 9. After 50 mg $MgSO_4$. All time scales = 100 msec.

[5] Philips, F. S., and Gilman, A., *J. Pharmacol.*, 1946, **86**, 213.

[6] Brown, G. L., and Harvey, A. M., *Brain*, 1939, **62**, 341.

[7] Köllensperger, F. K., *Klin. Wchnschr.*, 1940, **19**, 128.

mine accentuated the repetitive response. Conversely, after a larger dose of metrazol (50 mg/kg), which evoked intense repetition, the injection of neostigmine suppresed the usual response.

As a gauge of one phase of excitability, measurements were made in nerve of the rate of recovery of responsiveness, the relatively

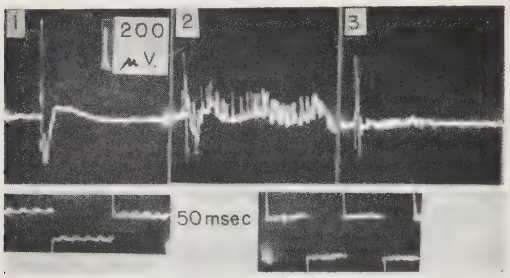

FIG. 3.
Electroneurograms. 1. Control. 2. After 10 mg DDT. 3. After 50 mg Ca gluconate.

refractory period, before and after treatment with metrazol. The results of 2 experiments have been grouped and are presented in Fig. 4. Concurrent with the appearance of repetition after administration of metrazol, the nerve recovered more rapidly and the relatively refractory state subsided more abruptly. In addition, a minimal but recognizable period of supernormality appeared.

Discussion. Evidence is accumulating slowly to support the hypothesis that agents which produce repetitive responses in one sort of excitable tissue induce generally the same altered state of excitability in other tissues of the same organism. An example of such a general effect is furnished by metrazol, which produces central rhythmic discharge and convulsions, and in the periphery evokes repetition in isolated nerve and striated muscle.

The same alteration of excitability resulting in repetition can be evoked in members of widely separated phyla. An example of this broad action is furnished by DDT which produces the same veratrinic response in the peripheral neuromuscular structures of both arthropods and mammals. DDT also fits into the group of substances which affect peripheral as well as several central structures.[8,9]

The changes which underlie the development of veratrinic repetition are obscure. The assumption that repetition occurs in a setting of exalted excitability is compatible with the demonstration of an accelerated rate of recovery demonstrated in nerve treated with metrazol or 2, 4-dichlorphenoxyacetate (2,4-D).[10] But the number of apparently unrelated agents which evoke repetition makes it difficult to construct an hypothesis encompassing all reported observations. A recent and attractive working hypothesis of repetition has emphasized the primary role of free and surface-bound calcium as a modulator of excitability in nerve.[11] Whether this engaging concept will explain repetition occurring in many

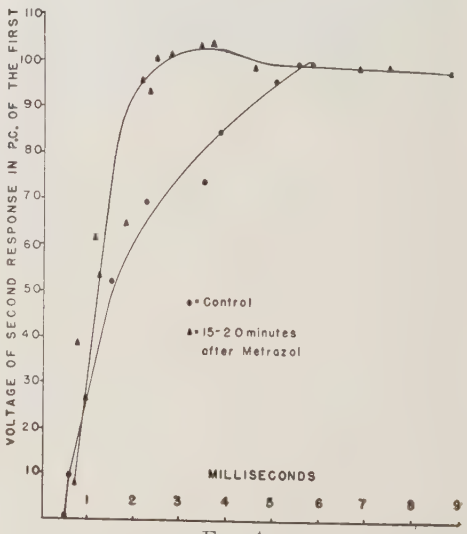

FIG. 4.
Relatively refractory period of nerve, measured before and after metrazol.

tissues and under many varying conditions is an unanswered question.

The development of repetition in the peripheral neuromuscular unit results in a functional disorder which is indistinguishable from

8 Crescitelli, F., and Gilman, A., *Am. J. Physiol.*, 1946, **147**, 127.

9 Bromiley, R. B., and Bard, P., *Bull. Johns Hopkins Hosp.*, in press.

10 Eyzaguirre, C., Jarcho, L. W., and Lilienthal, J. L., Jr., in preparation.

11 Gordon, H. T., and Welsh, J. H., *J. Cell. Comp. Physiol.*, 1948, **31**, 395.

the myotonia occurring spontaneously in man and goat. There is no evidence, however, to establish the identity of the induced and the spontaneous phenomena, similar though they appear. Nevertheless, the demonstrated effect of veratrinic agents on both nerve and muscle suggests that in spontaneous myotonia an as yet undetected repetition may occur in nerve as well as in muscle.

Summary. 1. Both metrazol and DDT evoke a veratrinic response in mammalian nerve and muscle.

2. The characteristics of this response are indistinguishable from those of myotonia occurring spontaneously.

3. The veratrinic effect of repetitive responses to single stimuli is accompanied by a shortening of the relatively refractory period.

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Comparison of Atropine and Tripelennamine in Treatment of Peptone Shock in Dogs.

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Dale and Laidlaw[1] first called attention to the close similarity existing between anaphylactic and peptone shock on the one hand and the type of shock produced by an injection of histamine on the other. Indeed, they postulated that histamine is the substance active in producing the classical signs of anaphylaxis. It was not until 1932, however, that their theory received substantiation, when Dragstedt[2] demonstrated the presence of a substance in blood and lymph in early stages of anaphylactic shock in dogs which resembled histamine chemically and physiologically. It is now generally accepted that histamine plays the major role both in peptone and in anaphylactic shock-like states.

Since the advent of potent anti-histaminic compounds, it has been demonstrated that all drugs which show a marked antagonism to histamine are capable of diminishing the severity of anaphylactic shock.[3] A number of observations, however, would indicate that some factor or factors other than histamine may be involved. For example, Went and Lissak[4] reported that the choline content of the isolated, sensitized heart of the guinea pig decreased following addition of antigen, and, moreover, that acetylation of the perfusion fluid evoked an acetylcholine-like reaction in leech muscle. Furthermore, the isolated, sensitized rodent heart slowed when antigen was added to the perfusing fluid. More recently, Farber and his co-workers[5] found that in 3 of 27 isolated, sensitized hearts (guinea pig) an acetylcholine-like substance was liberated to a perfusate containing physostigmine when the antigen was added. The *normal* heart failed to show the same response. Prior to this, Wenner and Buhrmester[6] reported the acetylcholine concentration in the blood of rabbits in anaphylactic shock to be 1:1,000,000 to 1:10,000,000, whereas measurable amounts were not detected in the blood of normal animals.

The relative importance of acetylcholine in the genesis of peptone and anaphylactic shock

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† Deceased June 28, 1948.

1 Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1910, **41**, 318.

2 Dragstedt, C. A., and Gebauer-Fuelnegg, E., *Am. J. Physiol.*, 1932, **102**, 512.

3 Loew, E. R., *Physiol. Rev.*, 1947, **27**, 542.

4 Went, S., and Lissak, K., *Arch. f. Exp. Path.*, 1936, **182**, 509.

5 Farber, S., Pope, A., and Landsteiner, E., Jr., *Arch. Path.*, 1944, **37**, 275.

6 Wenner, W. F., and Buhrmester, C. C., *J. Allergy*, 1937, **9**, 85.

TABLE I.
Pulse Rates Following Administration of Peptone.

Pretreatment	Dose I.V. mg/kg	Exp. No.	Before administ.	During administ.	2-5 min. post-administ.
Atropine • SO_4	2	1	204	180	208
		2	188	168	200
		3	188	176	176
		4	188	152	148
		5	192	180	208
		Avg	192	171	188
Pyribenzamine • HCl	10	1	164	160	180
		2	168	140	180
		3	130	144	140
		4	228	192	268
		5	152	148	160
		Avg	168	157	186
Atropine • SO_4 and Pyribenzamine	2 and 10	1	160	144	150
		2	190	140	208
		3	172	120	184
		4	160	152	188
		5	184	152	192
		Avg	173	142	184
Controls		1	172	160	192
		2	180	180	76
		3	184	144	96
		4	162	140	88
		5	160	140	72
		6	184	160	64
		Avg	174	154	98

has not been assayed, and the present report deals with attempts at a comparison of the beneficial effects of an antiacetylcholine agent and an antihistamine compound in peptone shock.

Methods. Mongrel dogs from a stock supply were anesthetized with sodium pentobarbital given by vein. The left carotid artery was exposed, cannulated, and connected with a U-tube mercury manometer modified for use with the electrical recording system described by Maison and Haterius.[7] Pulse rates were obtained by palpation. All drugs were administered via the femoral vein. Atropine sulfate, 2.0 mg/kg and Tripelennamine (pyribenzamine),‡ 10.0 mg/kg either alone or in combination were used as prepeptone medication. These were administered as 1.0% solutions. Ten to 15 minutes were allowed after the administration of pyribenzamine before peptone was given. Five cc (1.0 g) per kilo of a 20% solution of Witte's peptone was then injected intravenously as rapidly as possible; the time required for the injection was 1.5 to 3 minutes depending upon the total volume used.

Results. Mortality: Of 6 controls, receiving only peptone, 5 died within 5 to 11 minutes after beginning the injection. Of 6 dogs pretreated with atropine, 5 survived; of 5 receiving pyribenzamine all survived, and of 5 receiving both pyribenzamine and atropine there were no deaths. The statistical significance between control and pretreated dogs is great ($P = <.01$) but none exists between methods of pretreatment.

Pulse rates: During the peptone injection, the heart rate in most of the animals slowed 4 to 52 beats per minute from the initial rate. Within 2-5 minutes after the beginning of injection, the pulse rates of those dogs which were to survive increased to a level greater than that prior to administration of peptone. The controls developing fatal shock did not

[7] Maison, G. L., and Haterius, H. O., *J. Assn. Am. Med. Coll.*, 1947, **22**, 200.

‡ The Pyribenzamine was kindly supplied by Ciba Pharmaceutical Products, Inc.

exhibit this increase, but rather showed a constant decrease in rate until death. The changes in pulse rates are apparent from Table I.

Blood pressure: All of the dogs, regardless of the type of pretreatment, showed an initial

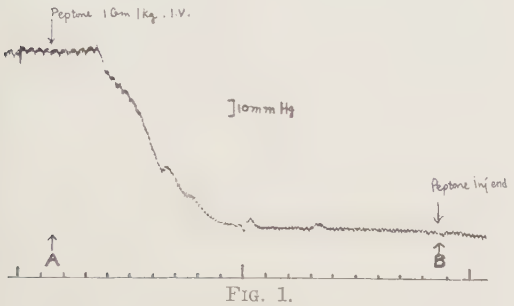

FIG. 1.

A typical example of the effect upon the blood pressure produced by the intravenous injection of peptone, 1.0 g/kg. Injection begun at A, completed at B. Time interval—6 seconds. Pressure at A, 140 mm Hg; pressure at B, 28 mm Hg.

precipitous fall in blood pressure to about 30 mm Hg beginning within 15 to 30 seconds after commencing the peptone injection. Fig. 1 is an example of this initial drop in blood pressure. Continuation of the injection evoked a further fall of only 5 to 10 mm in the treated animals, while the controls showed a progressive decline to zero. In a majority of the surviving animals the blood pressure began to rise in 4 to 10 minutes, reached a peak in 9 to 16 minutes, declined a few millimeters and then began a secondary more gradual rise within 20 to 30 minutes, reaching a plateau in about one hour. (All time intervals given refer to the time elapsed from the beginning of the peptone injection.) This initial rise, as well as the increase in pulse rates to greater than pre-peptone levels, is probably indicative of a generalized sympathetic discharge occurring in response to the profound hypotension. Six animals did not show the initial peak, but exhibited a slow continuous rise to the final plateau. Otherwise they were similar to the remaining animals. A typical example of the blood pressure changes for each of the 3 methods of pre-treatment is given in Fig. 2.

As pyribenzamine potentiates the response to epinephrine,[8] the blood pressure level at 25 minutes post-peptone, (at which time the pressure was at a plateau or just beginning its secondary rise) was used as an indication of the degree of protection afforded by the drugs, since this would minimize the effects of sympathetic discharge which were probably greatest somewhat earlier coincident with marked hypotension. Twenty-five minutes were arbitrarily chosen, because with further recovery the differences between the methods used became less marked. The blood pressure changes are shown in Table II.

None of the 3 methods of treatment prevented the initial fall in blood pressure. Both the height of the initial peak and the pressure at 25 minutes were greater in those treated

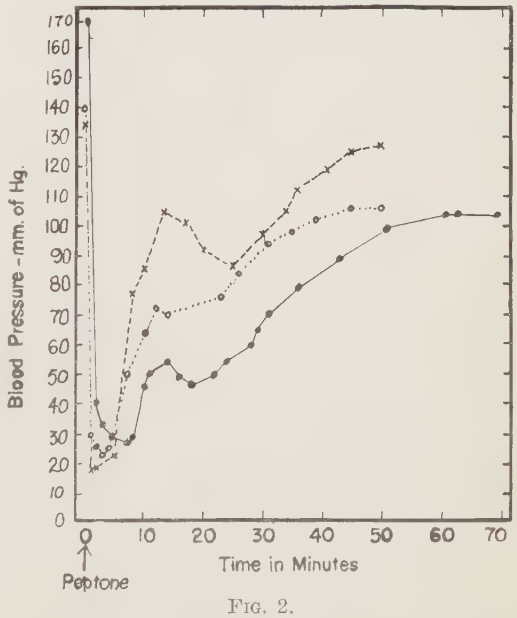

FIG. 2.

Typical blood pressure changes occurring after administration of peptone, 1.0 g/kg body weight. Pretreatment:

●——● Atropine sulfate, 2.0 mg/kg; Exp. No. 2.

x – – – – x Pyribenzamine • HCl, 10.0 mg/kg; Exp. No. 2.

○ • • • • ○ Atropine sulfate, 2 mg/kg, and Pyribenzamine • HCl, 10 mg/kg, Exp. No. 5.

with pyribenzamine alone than in those receiving atropine alone. In the dosages used all 3 methods of treatment protected dogs from death due to peptone. On the basis of blood pressure levels at 25 minutes after the adminis-

[8] Sherrod, T. R., Loew, E. R., and Schloemer, H. F., *J. Pharm. and Exp. Therap.*, 1947, **89**, 247.

TABLE II.
Blood Pressure Following Administration of Peptone.

Pretreatment	Dose I.V. mg/kg	Exp. No.	Blood pressure (mm Hg) Before administ.	2 min. after administ.	25 min. after administ.*
Atropine • SO_4	2	1	138	30	45
		2‡	170	34	56
		3	134	18	44
		4	112	18	10
		5	136	22	96
		Avg	138	24	50
Pyribenzamine • HCl	10	1	126	52	112
		2‡	142	20	88
		3	128	26	104
		4	152	36	76
		5	122	28	88
		Avg	134	32	94
Atropine • SO_4 and Pyribenzamine • HCl	2 and 10	1	80	28	106
		2	68	10	44
		3	144	24	56
		4	136	22	70
		5‡	136	24	80
		Avg	113	22	71
Controls		1	126	10	42†
		2	138	10	—
		3	150	18	—
		4	146	24	—
		5	140	16	—
		6	120	10	—
		Avg	137	15	—

* Statistical evaluation of atropine vs. pyribenzamine (t-test) shows P = .02.
† Only one of 6 controls survived, 5 died in 5 to 11 minutes as blood pressure reached O mm Hg.
‡ Data plotted in Fig. 2 as typical examples of each method of treatment.

tration of peptone, pyribenzamine afforded significantly better protection than atropine (P = .02). A combination of the two did not appear to afford better protection than pyribenzamine alone, as the rate of recovery from the hypotensive state was no greater in those receiving both drugs than in those receiving only pyribenzamine.

Discussion. If acetylcholine plays a role in the genesis of anaphylactic and peptone shock, pretreatment with a substance that blocks its action should lessen the severity of reaction. As long ago as 1910 Auer[9] reported atropine to be of value in anaphylactic shock, and obtained a 72% survival in guinea pigs pretreated with atropine before reinjection of the sensitizing serum, as compared with 25% survival in non-atropinized controls. Gross,[10] in finding 0.3 g/kg of Witte's peptone to be the MLD in dogs, reported atropine (0.2 mg/kg) would prevent death in all dogs subjected to 0.5 g/kg of peptone. Atropine in this dosage failed to protect against the lethal effects of 0.6 g/kg. The number of animals used was not stated.

On the other hand, in a report by Vallery-Radot, Mauric and Holtzer,[11] atropine in doses of 0.004 to 0.5 mg/kg did not prevent anaphylactic shock in rabbits. Of 7 animals, 2 (on a dosage respectively of 0.5 mg and 1.0 mg) did not exhibit shock. Despite this the authors concluded that atropine was ineffective in preventing this type of shock. Danielopolou,[12] however, reported atropine to hinder, and eserine to aid, the development of ana-

9 Auer, J., *Am. J. Physiol.*, 1910, **26**, 439.

10 Gross, E. G., *J. Pharm. and Exp. Therap.*, 1926, **30**, 351.

11 Vallery-Radot, P., Mauric, G., and Holtzer, A., *Compt. rend. Soc. de Biol.*, 1943, **137**, 18.

12 Danielopolou, D., *Acta. Med. Scandinav.*, 1944, **118**, 22.

phylaxis. Dosages were not given.

In the present instance, the failure to prevent the initial fall in blood pressure is consistent with the findings of Yonkman, Hays and Rennick[13] in anaphylactic shock. They reported, however, that the heart rate is apparently not increased during the hypotensive phase in anaphylaxis following pretreatment with pyribenzamine—a circumstance with which our results in the case of peptone shock are not in agreement.

One difficulty to be overcome before accepting the hypothesis that acetylcholine plays a role in the production of peptone shock is that, as yet, no drug is available which exhibits antagonism to acetylcholine without possessing some antihistaminic action. Similarly all antihistaminic drugs show a certain degree of antiacetylcholine activity. Various degrees of specificity are possible however. Thus pyribenzamine is 250 times as potent as atropine against histamine as tested by the aerosol method of producing histamine shock in guinea pigs.[14,15] Atropine is much more effective as an anticholinergic drug than is pyribenzamine. In our experience 10.0 mg/kg of pyribenzamine exerted only moderate vagal blockage, as compared with 0.05 mg/kg of atropine required for vagal blocking in the dog.[16]

To protect 100% of sensitized guinea pigs from lethal anaphylaxis, 0.3 mg/kg of pyribenzamine must be given.[15] The exact dosage required to protect dogs has not been determined, but it seems to be somewhat greater. Thus the protective action of 2.0 mg/kg of atropine in peptone shock can hardly be attributed to its antihistaminic properties. It seems much more likely that the benefit derived is a result of its anticholinergic activity.

Although it is generally accepted that histamine plays an important role in anaphylactic and peptone shock, it is possible that acetylcholine is also involved to some extent. The relative importance of histamine and acetylcholine remains to be determined more precisely. More quantitative comparisons between the antihistaminics and anticholinergic drugs are necessary before the question can be resolved, but the greater degree of protection afforded by pyribenzamine than by atropine, as shown in these experiments by the blood pressure changes, is evidence that while histamine plays the major role in the production of peptone shock, apparently acetylcholine is also concerned.

Conclusions. 1) One gram/kg body weight of Witte's peptone administered intravenously in a 20% solution, proved fatal in dogs anesthetized with sodium pentobarbital.

2) Atropine sulfate, 2.0 mg/kg prevented death from peptone.

3) Pyribenzamine, 10.0 mg/kg, afforded better protection than atropine as determined by rapidity of recovery of blood pressure.

4) The rapidity of recovery of blood pressure was not greater in those animals receiving both pyribenzamine and atropine than in those receiving pyribenzamine alone.

5) These findings support the theory that acetylcholine is concerned in the production of peptone shock, although histamine plays a more important role.

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[13] Yonkman, F. F., Hays, H. W., and Rennick, B., *Fed. Proc.*, 1944, **4**, 144.

[14] Halpern, B. N., *Arch. Internat. de Pharmacodyn. et de Therap.*, 1942, **68**, 339.

[15] Mayer, R. L., *J. Allergy*, 1946, **17**, 153.

[16] Sollmann, T. H., and Hanzlik, P. J., *Fundamentals of Experimental Pharmacology*, J. W. Stacey, Inc., San Francisco, 1939.

16898

Influence of Secretin and Insulin on Pancreatic Secretion in Healthy Human Subjects.

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The intravenous administration of highly purified secretin is known to stimulate the secretion of pancreatic juice which is low in both organic material and enzymatic activity. On the other hand vagus stimulation results in the secretion of pancreatic juice with a high enzyme concentration.[1] Central vagus stimulation induced by insulin hypoglycemia has been shown to be without effect on the secretion of fluid by the pancreas in both man[2] and the unanesthetized dog[3] providing adequate precautions are taken to exclude the acid gastric contents from the intestine. However, less consistent have been the studies on the influence of insulin hypoglycemia on the secretion of enzymes by the pancreas. Both inhibition[4,5] and excitation[6,7] of enzyme secretion have been reported.

Methods. The subjects comprising this study were 22 men and women who were either volunteers or clinic patients with no evidence of either gastro-intestinal or metabolic disease. About 15 to 18 hours after the previous meal the stomach and intestine were intubated with a single double-lumen tube of the type described by Agren and Lagerlof.[8,9] Exact location of the tube was always verified by fluoroscopy. Separate gastric and duodenal samples were collected by continuous aspiration at a negative pressure of 30 to 50 mm Hg.

The secretin used was prepared either in our laboratory or by Wyeth Incorporated by the procedure of Friedman and Thomas.[10] For the purpose of this study it is important to note that no evidence for the presence of pancreozymin[11] in this secretin preparation was found. The secretin had no effect on the blood sugar level, and did not stimulate either emptying of the gall-bladder or the secretion of intestinal juice.*

Following collection of a basal sample during a 20 minute control period the secretin was administered intravenously at a dosage level of 1.1 clinical units per kilogram body weight. Continuous collection of separate gastric and intestinal contents was carried out for the next 60 minutes, the samples being divided into the fractions obtained at the end of 10, 20, 40 and 60 minutes. A second standard dose of secretin was then given and collection of gastric and duodenal contents continued for the next hour. In one series of patients the second intravenous dose of secretin was combined with crystalline insulin, 0.1 unit per kilogram body weight. In some experiments a third injection of either secretin alone, or of secretin combined with insulin in the doses stated, was made at the end of the second hour and collection of digestive

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10 Friedman, M. H. F., and Thomas, J. E., to be published.

11 Harper, A. A., and Raper, S. S., *J. Physiol.,* 1943, **102**, 115.

* We are indebted to Dr. E. S. Nassett for the assay for enterocrinin.

TABLE I.

	1st hr Secretin			2nd hr Secretin		
	cc	Output	Conc. K	cc	Output	Conc. K
Highest	213	48140	.434	253	59720	.477
Lowest	82	7870	.072	127	9982	.072
Avg	143	22658	.158	186	29244	.157
Increase, %				30.1	29.1	0
	Secretin			Secretin + insulin		
Highest	283	67354	.238	287	57680	.288
Lowest	94	9580	.056	119	16070	.093
Avg	163	24910	.153	195	37812	.194
Increase, %				19.2	51.8	26.7

fluids continued during the third hour. Blood samples for sugar determinations were taken immediately before the combined injection of insulin and secretin and at 30 minutes and 60 minutes after the injection. All experiments involving the use of insulin were terminated by giving the subject dextrose solution through the gastric tube. Bicarbonate was determined by the titrometric procedure of Van Slyke.[12] Lipase concentration was determined on a tributyrin substrate by a phototurbidometric method,[13] and expressed in terms of percent standard substrate hydrolyzed by 0.1 cc of duodenal fluid in 20 minutes. Amylase concentration was determined by the picric acid method of Myers, Free and Rosinski.[14] Trypsin concentration was determined on an albumen substrate by the turbidometric method of Riggs and Stadie[15] as modified by Friedman.[16] Blood sugar concentration was determined by the Folin-Wu procedure.

Results. Following the injection of secretin the secretion of pancreatic juice reached its maximum rate during the second ten minute period and amounted to 82 to 213 cc (average 143 cc) during the hour (Table I). The concentration of enzymes was also greatest during the second 10 minute period but decreased thereafter at a more rapid rate than did the volume. In some instances the fluid recovered from the intestine during the last 20 minutes was pratically free from trypsin while amylase and lipase were still present in measurable concentrations.

A second dose of secretin in 22 experiments yielded from 127 to 253 cc (average 186 cc) of intestinal content during the hour, with the maximum rate of secretion usually occurring during the first 20-minute period. The secretory rate fell less rapidly during the hour following the second injection of secretin than during the hour following the first injection. The concentration of enzymes following the second secretin injection usually reached a maximum during the first 10 minute period. In all except 3 of the 22 experiments the concentration of enzyme following the second dose of secretin was less than that following the first dose (Table I).

In 17 other experiments the second secretin injection was combined with the injection of insulin. The blood sugar levels before insulin ranged from 66 to 93 mg %, 30 minutes after the administration of insulin from 23 to 48 mg %, and at the end of one hour from 30 to 105 mg %. In only one patient did the blood sugar level at the end of the hour exceed the initial value. During the first hour, when secretin alone was given, the volume secreted ranged from 94 to 283 cc (average 163 cc) while during the second hour, when insulin was combined with the secretin, the volume ranged from 119 to 287 cc (average 195 cc). The

12 Van Slyke, D. D., Stillman, E., and Culler, G. E., *J. Biol. Chem.*, 1919, **38**, 167.

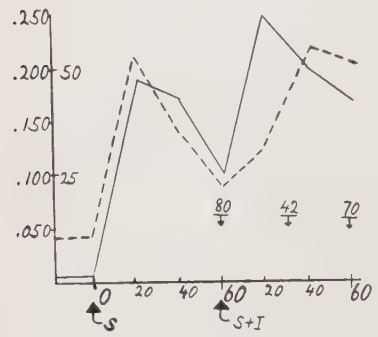
13 Friedman, M. H. F., to be published.

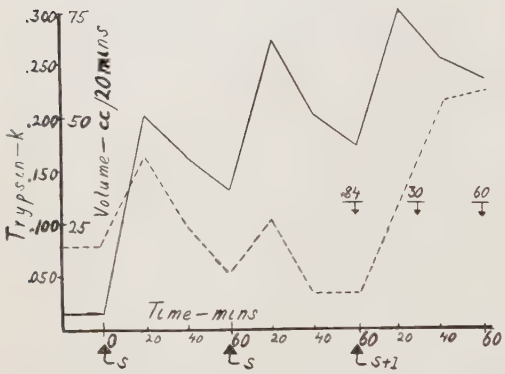
14 Myers, V. C., Free, A. H., and Rosinski, E. E., *J. Biol. Chem.*, 1944, **154**, 39.

15 Riggs, B. C., and Stadie, W. C., *J. Biol. Chem.*, 1943, **150**, 463.

16 Friedman, M. H. F., *Gastroenterol.*, 1947, **3**, 527.

rate of secretion following the administration of insulin combined with secretin was thus not different from that occurring following a second dose of secretin alone. However, in contrast with experiments in which secretin





Mr. L. L., psychoneurosis, no gastrointestinal disease demonstrated. Underscored figures in each graph represent mg % blood sugar before and after insulin. Solid line shows volume, dotted line shows trypsin concentration.

FIG. 1a (upper) Pancreatic response to a dose of secretin (S) followed one hour later by a dose of secretin combined with insulin (S + I).

FIG. 1b (lower). Pancreatic response to 3 hourly injections of secretin (S), the last one being combined with insulin (S + I).

alone was given, in each of these experiments the concentration of enzymes secreted during the second hour was higher than during the first hour. The average increase in output of enzymes was 51.8% (Table I).

In 6 experiments 3 hourly injections of secretin were given. In 3 of these experiments the third secretin injection was combined with insulin. Three consecutive hourly injections of secretin alone resulted in a fairly steady increase in rate of secretion with a progressive decrease in the concentration and total output of enzymes. Combination of insulin with the third secretin injection had little influence on the rate of secretion but resulted in a very definite increase in the enzyme output (Fig. 1).

Discussion. The intravenous administration of secretin to the healthy fasting subjects evoked the typical pancreatic response attributed to the hormone. After a second dose of secretin the volume of juice secreted was greater than after the first dose, probably due to the cumulative effects of both secretin injections. After each injection of secretin the concentration of enzymes gradually diminished following an initial transitory rise. Since secretin is believed to excite active secretion of bicarbonate and water only, but not of enzymes, the initial transitory increase in enzyme concentration has generally been attributed to the washing out of the enzyme from the acinar cells and ducts by the flow of water. According to some investigators[17] a constant synthesis of protein material would make available new enzyme substances which would be similarly washed out by the flow of water that is put into operation by a second dose of secretin. Experimental evidence for this process, however, is still wanting.

Another explanation has been advanced by Thomas[18] and recently by Wang *et al.*[19] An increase in the dose of secretin, as well as a new injection of a submaximal dose of secretin, as was done in our experiments, may bring previously resting secretory elements into activity. This would result in the initial transient increase in enzyme output after each secretin injection.

Compared to the effects of secretin alone, the addition of insulin to the secretin did not influence the volume response. However, the output of enzymes by the pancreas secreting in response to secretin was increased by about 60% when a hypoglycemic state was induced by means of insulin. It is well-known that vagus excitation may stimulate the se-

[17] Komarov, S. A., Langstroth, G. O., and McRae, D. R., *Canadian J. Research*, 1939, **17**, 113.

[18] Thomas, J. E., *Fed. Proc.*, 1942, **1**, 261.

[19] Wang, C. C., Grossman, M. I., and Ivy, A. C., *Am. J. Physiol.*, 1948, **154**, 358.

cretion of pancreatic juice of high enzyme concentration, and that insulin hypoglycemia may excite vagus centers. The latter has been proved abundantly by studies on gastric secretion and motility. It may be assumed that the excitatory effect of insulin on the secretion of pancreatic enzymes is due to a generalized hypoglycemic excitation of visceral centers, including those of the vagus.

Summary. Healthy human subjects were given 2 or 3 hourly intravenous injections of secretin. In some of the experiments insulin was given intravenously during the course of the pancreatic secretion evoked by the secretin. It was found that during insulin hypoglycemia the concentration and output of pancreatic enzymes was increased but that the volume of secretion was not influenced.

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Heat Stability of Hemagglutinin of Various Strains of Newcastle Disease Virus.*

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Determination of the heat stability of the hemagglutinin was selected as the first method of approach to the problem of characterizing the antigenically homologous strains[1] of Newcastle (NDV) virus. A method of identifying strains was deemed necessary for proposed studies of virulence and immunogenicity of the virus. For purpose of survey, 24 strains of virus obtained from widely distributed points in North America and Europe were cultured in embryonating eggs and the stability of the hemagglutinin (HA) to heating at 56°C determined.

The heat stability of the influenza hemagglutinin[2] has been extensively studied. It was the demonstration by Salk[3] of differences in heat stability of the influenza strains which influenced our choice of approach. Scott and Lauffer[4] described the inactivation of hemagglutinin of influenza virus as a first order reaction by assuming a multicomponent system. The demonstration that the site of inactivation of the hemagglutinin and the site of infectivity for embryos were independent was, therefore, not surprising.[5] The Henles[6] have shown that with heat and ultraviolet treatment, certain properties of influenza virus can be arranged in a descending order of susceptibility to inactivation. The capacity to infect embryos was lost first, then, in order, the ability to interfere with infection, to elute from agglutinated red blood cells, to agglutinate red blood cells, and subsequently, the ability to fix complement in the presence of immune sera. Antigenicity could not always be fitted into this order, but sometimes it persisted until after the hemagglutinin had been destroyed. With certain strains of influenza virus Salk[7] demonstrated the protec-

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1 Brandly, C. A., Moses, H. E., Jungherr, E. L., and Jones, E. E., *Am. J. Vet. Res.*, 1946, **7**, 289.

2 Burnet, F. M., *Aust. J. Exp. Biol. and Med. Sci.*, 1942, **20**, 81.

3 Salk, J. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 134.

4 Scott, E. M., and Lauffer, M. A., *Arch. Biochem.*, 1946, **11**, 185.

5 Lauffer, M. A., Carnelly, H. L., and MacDonald, E., *Arch. Biochem.*, 1948, **16**, 321.

6 Henle, W., and Henle, G., *J. Exp. Med.*, 1947, **85**, 347.

7 Salk, J. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 140.

TABLE I.
Description of Strains.

Place of isolation	Date of isolation	Donor	Source animal	Code
New Jersey	1944	F. R. Beaudette	Chicken	N. J. (NJ-KD)
?	?	F. R. Beaudette	?	Beaudette vaccine (FRB)
New York	45	P. P. Levine	Chicken	N. Y. Levine (NYL)
New York	46	J. Fabricant	Turkey	Mayer turkey (NYT)
Massachusetts	45	C. A. Brandly	Chicken	Huntington Lab (4F)
Iowa	46	M. A. Hofstadt	Chicken	23 E 219 (HOF)
Michigan	46	C. H. Cunningham	"	46-967 (MIC)
Missouri	47	H. C. McDougle	"	31747 (31747)
Kansas	48	L. D. Bushnell	"	Leavenworth (KL)
Kansas	48	L. D. Bushnell	"	Manhattan (KM)
Wisconsin	46	Field	"	Lancaster 1660 (WL)
Wisconsin	48	Field	Turkey	Henry 24156 (HHT56)
Minnesota	47	B. S. Pomeroy	"	(TM)
California	44	J. R. Beach	Chicken	(11914)
Canada	47	R. V. L. Walker	"	Allen M4947 (ALL)
"	47	R. V. L. Walker	"	Berwick 29507 (BER)
"	47	R. V. L. Walker	"	Cleroux M4969 (CLE)
"	47	R. V. L. Walker	"	Herr M5004 (HER)
"	47	R. V. L. Walker	"	Labelle M4970 (LAB)
"	47	R. V. L. Walker	"	McVey M5016 (McV)
"	47	R. V. L. Walker	"	Watford No. 5 (WAT)
"	47	R. V. L. Walker	Turkey	Peerless M4945 (TP)
England	33	Min. of Agric.	Chicken	Hertfordshire (E)
Italy	45	U. S. Army	"	Milano (M)

tive action of traces of formalin to the denaturation process. Francis[8] reported the divergence of the capacity to show inhibition in the presence of immune sera when the hemagglutinin was heated.

Methods. For ease of comparison and for the sake of simplicity the methods used follow those for similar work with influenza virus insofar as the properties of Newcastle virus permit.

Virus material from an early passage of each strain (less than 10 egg passages) was utilized to avoid changes which may develop in the character of the strain on prolonged cultivation of Newcastle virus in the embryonating egg.[1,9-12] Only freshly harvested virus or material held for less than one month at —20°C was used for determinations. Allanto-amnionic fluids become less stable to heat treatment after storage at 4°C, but no significant changes were observed after storage at —20°C for the periods and strains studied.

Clear allanto-amnionic fluids from moribund or dead embryos were sealed in 5 ml lyophilizing vials by drawing out the necks of the vials in a flame. The vials were submerged in a constant temperature water bath at 56 ± 0.5°C, the water being agitated constantly with an electric stirrer. Vials were removed at selected intervals and immediately chilled in ice water. Pooled allanto-amnionic fluids from four to eight eggs were used for all determinations. Heat stability data were obtained on at least two pools and in most instances four or five pools of each strain. Virus pools of a given strain, but not necessarily individual egg fluids of a given strain, gave closely reproducible results. Reasons for individual egg variations have been explored, and with certain precautions observed in har-

[8] Francis, T., Jr., *J. Exp. Med.*, 1947, **85**, 1.

[9] Iyer, S. G., and Dobson, N., *Vet. Rec.*, 1941, **53**, 381.

[10] Iyer, S. G., and Hashni, Z. A., *Indian J. Vet. Sci.*, 1945, **15**, 155.

[11] Winslow, N. S., 1947. Thesis submitted for degree of Master of Science, University of Wisconsin.

[12] Beaudette, F. R., Proc. 47th Ann. Meet. U. S. Livestock San. Assoc., 1943, 122.

TABLE II.
Heat Stability of the Hemagglutinin at 56°C.

Strain	Minutes*						
	0	15	30	60	120	240	480
NJ-KD	1280	0	0	0	0	0	0
FRB	1280	0	0	0	0	0	0
NYL	1280	20	0	0	0	0	0
NYT	1280	10	0	0	0	0	0
4F	1280	160	10	0	0	0	0
HOF	1280	160	20	0	0	0	0
MIC	1280	640	40	0	0	0	0
31747	1280	320	40	0	0	0	0
KL	1280	1280	1280	1280	320	160	0
KM	1280	1280	1280	640	320	5	0
WL	1280	40	0	0	0	0	0
HHT56	1280	0	0	0	0	0	0
TM	1280	1280	640	640	160	10	0
11914	1280	320	160	10	0	0	0
ALL	1280	1280	1280	640	320	160	0
BER	1280	1280	1280	640	320	20	0
CLE	1280	1280	1280	640	80	20	0
HER	1280	1280	1280	1280	640	160	0
LAB	1280	1280	640	640	320	10	0
McV	1280	640	320	160	20	0	0
WAT	1280	1280	640	40	0	0	0
TP	1280	1280	1280	640	320	40	0
E	1280	160	20	0	0	0	0
M	1280	1280	1280	640	80	20	0

* Data obtained at different time intervals (see text) are not included in table.

vesting fluids, close correlation can be obtained among individual egg fluids containing virus of a given strain.

The virus pools ranged between pH 7.9 and pH 8.2. There was no relationship between pH of the fluids on harvest and stability of the virus in the fluid to heating.

The hemagglutination test procedure substantially followed that outlined by Salk.[13] A 0.2 ml volume of virus was added to 0.8 ml of saline in agglutination tubes (14.5 × 75 mm). Five-tenths of a milliliter of this 1:5 dilution was transferred to 0.5 ml of saline, thoroughly mixed, and similar transfers repeated until a 1-1280 dilution was obtained. Twenty-five hundredths milliliters of 1% fowl red blood cells was added to all tubes and the rack shaken well. The hydrogen ion concentration of the virus-saline-red cell mixtures ranged from pH 7 to 8. The test was incubated at room temperature of 25 to 30°C and read from 15 to 45 minutes later,[14] the period of reading being determined by the controls. The endpoint was considered to be a definite one plus agglutination. The effect on titers of differences in the agglutinability of red cells from one fowl to another was considerably resolved by using the highly agglutinable cells of a single fowl as a standard for all tests.[14]

Experimental. A description of the 24 strains is given in Table I. The localities of isolation are roughly grouped into 5 regions, eastern United States, the middle western United States, western United States, Canada, and Europe. The dates of isolation range from 1933 to 1948, a period of 15 years. Four of the strains were obtained from turkeys and 20 from chickens. The abbreviated code given in parentheses following the complete strain designation is used in the text and accompanying figures.

[13] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

[14] Brandly, C. A., Hanson, R. P., Lewis, S. H., Winslow, N. S., Hoyt, H. H., Pritchard, W. R., and Nerlinger, C. M., *Cornell Vet.*, 1947, **37**, 324.

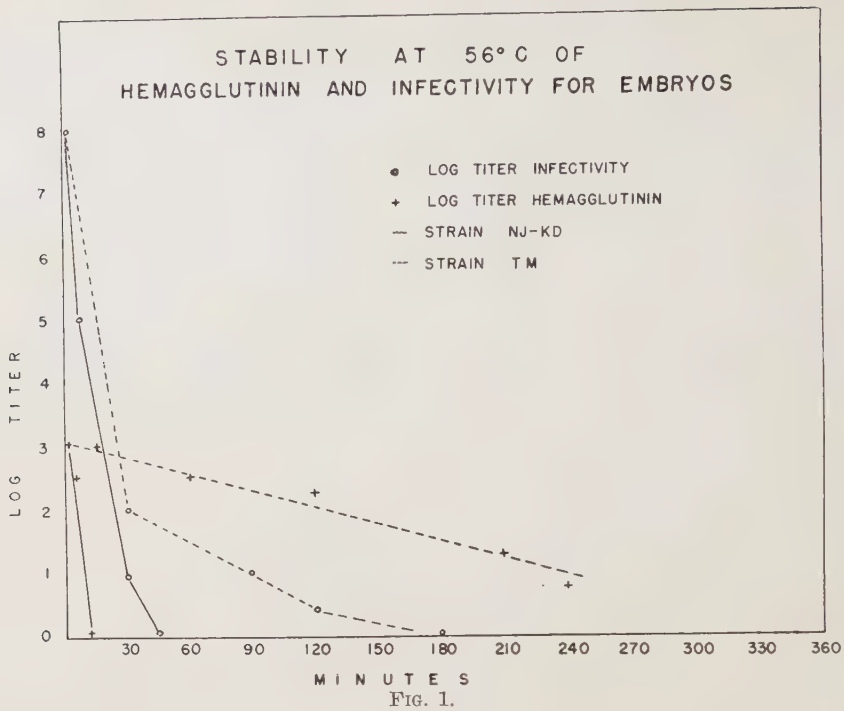

FIG. 1.

The stability of the hemagglutinins is depicted in Table II. Six periods at geometrically increasing intervals, beginning with 15 minutes and ending with 240 minutes, define the stability range of the hemagglutinins of the virus strains observed. The titer of all strains before treatment was approximately 1-1280, which is the usual titer obtained with red blood cells from selected fowls utilized in this study. Three of the 24 strains lost the power to agglutinate red cells within 15 minutes of heat treatment. Of these, FRB and HHT56 had low titers and NJ-KD no titer at all at 10 minutes. At 5 minutes, however, NJ-KD had a significant titer. Six of the 24 strains showed no hemagglutinins at 30 minutes heating. By 60 minutes the number of strains failing to agglutinate erythrocytes increased to 11 and after 2 hours heating to 13. Four hours of heating destroyed the agglutinin of all but 10 of the 24 strains. Four strains, KL, Ber, Cle and M, persisted for 6 hours. No agglutinins have ever been observed after 8 hours of heating at 56°C.

Immediately the question is asked: Is the embryo infectivity (EI) inactivated at the same rate as the hemagglutinin? Fig. 1 presents exploratory data that can be explained only by assuming distinct inactivation rates.

Discussion. Great variation in the stability of the hemagglutinin of individual Newcastle virus strains is apparent from the data given in Table II. Additional differences can be best shown by certain comparisons with studies made on influenza virus strains. Lauffer[5] presented evidence that the site of inactivation of the hemagglutinin and the infectivity for embryos was quite distinct. That Newcastle virus hemagglutinin and infectivity for embryos are inactivated independently is shown in Fig 1. Significant is the fact that demonstrable hemagglutinin of the NJ-KD strain is destroyed before demonstrable infectivity for embryos is lost. The infectivity of the TM strain, on the contrary, is inactivated more rapidly than the hemagglutinin. Most strains having hemagglutinins of low heat stability resemble NJ-KD in their HA/EI stability and most strains having hemagglutinins of high stability resemble TM.[15] Apparently, infectivity of the virus

[15] Upton, E., 1948. Thesis submitted for degree of Master of Science, University of Wisconsin.

for embryos has a narrower range of heat stability than the virus hemagglutinin. The former ranged from 30 to 180 minutes, as compared to 5 to 360 minutes for the latter. The descending order of susceptibility to heat treatment obtained by the Henles for certain activities of influenza virus is not paralleled with Newcastle virus. With the latter either activity, the HA or the EI, may be destroyed first.

The ability of the virus to fix complement in the presence of immune serum is very stable under heat treatment, withstanding 100°C for at least an hour. Using only three strains, 0.01% formalin failed to increase the stability of the hemagglutinin. The possibility still remains that certain other Newcastle virus strains, like some of the influenza strains, may have hemagglutinins more stable to heat in the presence of traces of formalin.

Comparisons of heat resistance of the hemagglutinating activity of the strain with the locality from which it was isolated suggests that a survey, based on many strains, may show a close correlation between them, especially if the time of isolation is considered. All five strains obtained from the eastern seaboard of the United States were of low heat resistance, 4 having either very low or no stability at 15 minutes. All 8 Canadian strains were highly heat resistant, 7 of them having hemagglutinins persisting for 2 to 4 hours. The strains from the Middle West showed more variation in their stability. When the time of isolation of these strains is taken into consideration, it is noted that of 3 obtained in 1946, all were of medium resistance; of 2 obtained in 1947, 1 was of medium and 1 of high resistance; and of 3 obtained in 1948, 1 was of low and 2 were of high resistance. It is possible that a shift in the heat resistance of the midwest strains is occurring and that it might be related to mutation of existing strains or to the introduction of new strains. The existence of such a possibility merits further investigation by this and other methods of strain characterization.

Conclusion. The stability of the hemagglutinin of different Newcastle disease virus strains, when subjected to increased temperatures, has been found to vary over a wide range. The stability of the hemagglutinin of 1 isolate was destroyed at 56°C in a period as short as 5 minutes, and that of another was diminished only after 6 hours exposure. The stability of the hemagglutinin of Newcastle virus is discussed in relationship to influenza viruses. A relation of heat stability of strains to place and time of isolation is observed.

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Comparative Nutritive Value of Butter and Vegetable Fats Under Conditions of Low Environmental Temperature.*

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It has been generally accepted by nutritionists that, aside from differences in vitamin potencies, animal and vegetable fats have essentially the same nutritive value. On diets containing an adequate amount of B

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Contribution No. 208 from the Department of Biochemistry, University of Southern California.

TABLE I.
Comparative Effects of Butter and Vegetable Fats on the Gain in Body Weight of Immature Rats Maintained Under Cold Room and Room Temperature Conditions.

Dietary fat	No. of rats	Initial body wt., g	Gain in body wt 8 wk period,* g
	Cold room series		
Cottonseed oil	10	65.5	110.0 ± 3.8
Corn oil	10	65.1	100.4 ± 6.4
Margarine fat	10	65.3	94.7 ± 4.2
Butter fat	10	64.4	100.6 ± 4.6
	Room temperature series		
Cottonseed oil	6	64.3	129.7 ± 4.1
Corn oil	6	64.7	138.3 ± 7.9
Margarine fat	6	64.7	119.7 ± 6.5
Butter fat	6	64.2	139.8 ± 9.3

* Standard error of the mean $\sqrt{\frac{\epsilon d^2}{n-1}} / \sqrt{n}$

vitamins no significant difference in growth has been observed under room temperature conditions in immature rats fed diets containing butter fat or various vegetable oils.[1-3] Available data indicate, however, that requirements for essential nutrients may be significantly increased by exposure to cold and other conditions of "stress."[4] It was felt that if differences exist in the nutritive value of fats, these might be accentuated under the stress of low environmental temperature. In the present study immature female rats were raised to maturity on purified rations containing butter and vegetable fats and their rate of growth determined under conditions of low environmental temperature.

Procedure and Results. Four experimental rations were employed in the present experiment, differing only in the source of fat. These consisted of 53.5% sucrose, 22.0% casein,† 4.5% salt mixture,‡ and 20.0% of either cottonseed oil, corn oil, margarine fat or butter fat.§ To each kg of the above diets were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2-methyl-naphthoquinone 5 mg and choline chloride 1.2 g. Each rat also received once weekly a vitamin A-D concentrate‖ containing 150 U.S.P. units of vitamin A and 15 U.S.P. units of vitamin D together with 3 mg of alpha-tocopherol acetate. Seventy-two female rats of the Long-Evans strain were selected at 28 to 30 days of age and an average weight of 64.9 g for the present experiment. Animals were placed in individual metal cages with raised screen bottoms to prevent access to feces, and were fed the above diets *ad lib.* Feeding was continued for 8 weeks. Experiments were conducted (1) with animals kept continuously in a large walk-in refrigerator at a temperature of 2±1.5°C, and (2) under standard labora-

[1] Boutwell, R. K., Geyer, R. P., Elvehjem, C. A., and Hart, E. B., *Arch. Biochem.*, 1945, **7**, 143.

[2] Deuel, H. J., Jr., Movitt, E., Hallman, L. F., and Mattson, F., *J. Nutrition*, 1944, **27**, 107.

[3] Deuel, H. J., Jr., Greenberg, S. M., Savage, E., and Fukui, T., unpublished data.

[4] Ershoff, B. H., *Physiol. Rev.*, 1948, **28**, 107.

† Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

‡ Sure's Salt Mixture No. 1,[5].

[5] Sure, B., *J. Nutrition*, 1941, **22**, 499.

§ The fats were obtained from the following sources: Cottonseed oil, Wesson Oil, and Snowdrift Sales Co., New Orleans, La., corn oil, Corn Products Refining Co., Argo, Ill.; margarine fat, Best Foods, Inc., New York, N. Y.; butter fat, Knudsen Creamery Co., Los Angeles, Calif. The butter and margarine were melted, and the water and protein separated by centrifugation. The fat was poured off and mixed to give a homogenous sample.

‖ Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

TABLE II. Summary Table Showing Average Gain in Weight, Average Food Consumption and Ratio of Increase in Weight to Calories Consumed for the First 4 Weeks of Feeding.

Dietary fat	Avg gain in body wt,* g	Food consumption (g/day) on following wks of experiment 1st	2nd	3rd	4th	Avg total food intake per rat for first 4 wks of feeding‡† g	Calories	Efficiency‡
				Cold room series (10 animals per group).				
Cottonseed oil	62.4 ± 2.9	11.7	13.2	13.4	14.8	371.7 ± 9.3	1784.2 ± 44.6	3.50 ± .10
Corn oil	62.4 ± 4.3	11.4	13.0	13.0	15.2	368.2 ± 9.7	1767.4 ± 45.0	3.54 ± .18
Margarine fat	58.9 ± 3.4	11.3	13.1	13.6	14.2	365.4 ± 11.0	1753.9 ± 50.0	3.36 ± .05
Butter fat	63.0 ± 3.1	11.3	13.6	14.2	15.1	379.4 ± 10.0	1821.1 ± 49.8	3.46 ± .13
				Room temperature series (6 animals per group).				
Cottonseed oil	86.3 ± 2.6	9.6	10.5	11.3	11.6	301.0 ± 6.9	1444.8 ± 33.0	5.97 ± .19
Corn oil	91.5 ± 5.8	8.9	11.4	11.4	12.3	308.0 ± 14.7	1478.4 ± 70.4	6.19 ± .16
Margarine fat	83.7 ± 2.9	10.0	12.3	13.0	12.8	336.7 ± 11.9	1616.2 ± 57.2	5.18 ± .15
Butter fat	93.3 ± 8.7	9.4	11.0	12.0	10.8	302.4 ± 21.0	1451.5 ± 100.8	6.42 ± .28

* Standard error of the mean; see footnote to Table I.
† The calorie value of these rations was approximately 4.8 calories per gram of diet.
‡ $\frac{\text{G increase in weight}}{\text{Calories consumed}} \times 100$

tory conditions at an average temperature of approximately 21±2°C. The cold room groups consisted of 12 rats each; the room temperature groups of 6. Food consumption was determined for each rat during the first 4 weeks of feeding. All diets were prepared weekly and kept under refrigeration when not in use.

The findings are summarized in Table I. Data for the cold room series were computed on the basis of the top 10 animals in each group to minimize variations in averages due to early deaths, infection, and atypical responses on the part of individual rats. Growth was markedly reduced in all rats under cold room conditions. Gain in body weight was most marked on the cottonseed oil diet and least on the ration containing margarine fat. In the room temperature series gain in body weight was most pronounced on the butter fat diet and least for margarine fat. The differences in growth on the various rations, however, either under cold room or room temperature conditions, were not sufficiently marked to be statistically significant.

Data on food consumption and the relative efficiency of the various diets for the building of body tissues are summarized in Table II. The findings indicate that for the first 4 weeks of feeding both under cold room and room temperature conditions virtually no difference in relative efficiency as measured by the ratio of gain in weight × 100 to the calories consumed was obtained for diets containing cottonsed oil, corn oil, or butter fat. Values were somewhat lower in the case of margarine fat, but in view of the small number of animals employed it is questionable whether these differences are significant. Values for the cold room series were less than for the room temperature series, a finding due, at least in part, to the increased metabolism of animals maintained under cold room conditions.

The findings indicate that butter fat, margarine fat, cottonseed oil, and corn oil have substantially the same nutritive value as judged by gain in body weight and efficiency of food utilization of young rats maintained at low environmental temperatures and fed diets differing only in the source of dietary fat.

Summary. Immature female rats were

raised to maturity under cold room and room temperature conditions on purified rations differing only in the source of fat. The fats employed were cottonseed oil, corn oil, margarine fat, and butter fat. Gain in body weight was significantly reduced in all rats under cold room conditions. No significant difference was observed, however, either under cold room or room temperature conditions, in gain in body weight on the various diets employed.

16901

Central Inhibitory Effects of Carbon Dioxide. II. *Macacus rhesus.*

S. N. STEIN AND G. H. POLLOCK. (Introduced by W. S. McCulloch.)

From the Department of Psychiatry, University of Illinois, Illinois Neuropsychiatric Institute.

Pollock's observation that carbon dioxide prevents electrically induced cortical seizures in cats suggested further study on primates.[1] Since the normal electroencephalograph of the *Macacus rhesus* is so well known, and it is not difficult to extrapolate from it to the human, this animal was chosen for the primate study.

Tracheal intubation, exposure of both femoral veins and of the calvarium were performed under ethyl ether anesthesia. Artificial respiration was started after an initial intravenous injection of 20 mg of dihydro-B-erythroidin hydrobromide and was maintained at 45 cc per stroke, 30 strokes per minute. 180 mg of dihydro-B-erythroidin hydrobromide in 300 cc of normal saline was administered as a slow drip throughout the experiment. Bilateral screw-in electrodes were placed 4 mm posterior to the coronal suture and 6 mm lateral to the sagittal suture. These electrodes were connected to a double-pole-double-throw switch allowing alternate connections to the input of a Goodwin non-blocking EEG amplifier with ink-writing oscillograph or to the output of a Lab-Tronics Stimulator (Model 3). The EEG was recorded concurrently with the EKG picked up from the right fore and left hind limbs.

Routinely the animals were ventilated with various concentrations of carbon dioxide in oxygen for intervals of 1 to 9 minutes and, on termination of each period of respiring carbon dioxide, stimulated with threshold and superthreshold voltages. A "saw-tooth" stimulating frequency of 60 impulses per second and a falling phase of 10 sigma were used throughout the experiment. It was found that concentrations of carbon dioxide below 15%, when administered for 3 minutes, would not prevent electrically-induced cortical seizures. When these concentrations were given for periods longer than 3 minutes, suppression of seizures occurred, which was more marked as the concentration of carbon dioxide approached 15%. Concentrations of carbon dioxide over 20% prevented the seizure response. Duration and intensity of the stimuli did not markedly affect the result. It appears that in *Rhesus macacus* the anticonvulsive level of carbon dioxide lies somewhere between 15% and 20%. The changes in the EEG and EKG found with carbon dioxide were similar to those found in cats by Pollock.[1]

Lorente de No' found that the rise of the membrane potential due to carbon dioxide is roughly proportional to the logarithm of its concentration, and this was accompanied not only by a decrease in fatiguability but also by a rise in threshold.[2] It is also known that carbon dioxide alters the pH, oxygen tension and blood flow of the brain.[3] Hence it is to be

[1] Pollock, G. H., Thesis, Univ. of Ill., College of Medicine, 1948.

[2] Lorente de No', R., Studies from the Rockefeller Institute for Medical Research, Vol. 131.

[3] Roseman, E., Goodwin, C. W., and McCulloch, W. S., unpublished data.

expected that with higher concentrations of carbon dioxide stronger stimuli would be required to evoke responses.

How these factors combine, and how carbon dioxide affects the action of other convulsants are being studied now.

Summary. Monkeys fitted with bilateral screw-in electrodes and immobilized with dihydro-beta-erythroidine were artificially ventilated with various concentrations of carbon dioxide-oxygen for different periods of time. At the end of these periods they were stimulated with threshold and super-threshold voltages, and it was found that concentrations of carbon dioxide over 20% prevented seizures when inhaled for 3 minutes.

16902

Central Inhibitory Effects of Carbon Dioxide. III. Man.

G. H. POLLOCK, S. N. STEIN, AND K. GYARFAS. (Introduced by W. S. McCulloch.)

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Previous investigations have shown that inhalation of carbon dioxide prevents electrically induced cortical seizures in cats[1] and monkeys.[2] This study was undertaken to note whether this phenomenon was also seen in man.

The subjects chosen were 18 carefully selected patients of varying ages. Fourteen were neurotics and the remaining 4, psychotics.* None had physical disability. The nature of the investigations were explained to them or their relatives prior to their volunteering for the test.

Each patient inhaled a commercially prepared mixture of known carbon dioxide concentration in oxygen for a definite period of time. At the end of this time an electrical stimulus sufficient to cause convulsion was given for 0.5 sec. from on Offner electroshock apparatus. As control, this same electrical stimulus was given after 5 minutes without carbon dioxide. Electroencephalograms were recorded with the Goodwin nonblocking EEG amplifier with ink-writing oscillograph.

The electrical stimulus without carbon dioxide resulted in grand mal seizures. It was found that concentrations of carbon dioxide from 15% to 30% routinely prevented the electrically induced seizures. With 30% carbon dioxide in oxygen, 30 seconds of inhalation sufficed. Slightly longer periods were required with 15 and 20%. With these mixtures of carbon dioxide several cases had decerebrate seizures, which will be fully described by Gyarfas *et al.*[3]

Haldane and Smith[4] found that 10% CO_2 was the upper limit which man could breathe without becoming unconscious. Lennox and Cobb[5] stopped petit mal seizures by having patients breathe high concentrations of CO_2 in air. Both show the central depressant action of CO_2 in man. Heinbecker[6] and Bishop,[7] Necheles and Gerard,[8] Hettwer[9] and

1 Pollock, G. H., Thesis, Univ. of Illinois College of Medicine, 1948.

2 Stein, S. N., and Pollock, G. H., PROC. SOC. EXP. BIOL. AND MED., in press.

* Thanks are due to the Chicago State Hospital for help with the selection of some of these patients and assistance in contacting relatives and arranging for voluntary transfers to Illinois Neuropsychiatric Institute.

3 Gyarfas, K., Pollock, G. H., and Stein, S. N., PROC. SOC. EXP. BIOL. AND MED., in press.

4 Haldane, J., and Smith, J. L., *J. Path. and Bact.*, 1892, **1**, 168.

5 Lennox, W. G., and Cobb, S., *Medicine*, 1928, **7**, 105.

6 Heinbecker, P., *Am. J. Physiol.*, 1929, **89**, 58.

7 Heinbecker, P., and Bishop, G. H., *Am. J. Physiol.*, 1931, **96**, 613.

8 Necheles, H., and Gerard, R. W., *Am. J. Physiol.*, 1930, **93**, 318.

9 Hettwer, J. P., *Am. J. Physiol.*, 1938, **122**, 275.

Lorente de No'[10] found that CO_2 raised the threshold of axons of the somatic and the autonomic nervous systems. Lorente de No'[10] showed that the carbon dioxide raised the membrane potential and lengthened the refractory period of nerve. The central inhibitory effects of the carbon dioxide may well be due to the increase of membrane potential and threshold. Moreover, breathing carbon dioxide increases cerebral blood flow by dilating cerebral arterioles, increases respiratory rate and so the oxygenation of blood, shifts the hemoglobin dissociation curve so that the blood releases more oxygen to the tissues and shifts cortical pH to the acid side. In the explanation of the central inhibitory action of the carbon dioxide these factors must all be considered.

Summary. Eighteen patients were ventilated with various concentrations of carbon dioxide-oxygen for varying lengths of time and then were stimulated with super-threshold shocking current. Concentrations of carbon dioxide from 15-30% routinely prevented electrically induced seizures; with 30% carbon dioxide, 30 seconds of inhalation sufficed; with 15-20% mixtures, slightly longer periods of time were required.

10 Lorente de No', Raphael, Studies of the Rockefeller Institute, vol. 131, 1937, New York.

16903

Central Inhibitory Effects of Carbon Dioxide. IV. Convulsive Phenomena.

K. GYARFAS, G. H. POLLOCK, AND S. N. STEIN. (Introduced by W. S. McCulloch.)

From the Department of Psychiatry, University of Illinois, College of Medicine, and Illinois Neuropsychiatric Institute.

Convulsive phenomena produced by inhalation of carbon dioxide were first mentioned by Waters.[1] Meduna and Gyarfas[2] observed 3 types of convulsive manifestations. The first type is the socalled adversive seizure which consists of conjugate deviation, extension of the arm and flexion of the leg on the side toward which the eyes turn. These fits are not accompanied by any change in the peripheral reflexes and can be explained as tonic neck reflexes due to premotor release.

The second type consists of flexion in the upper with extension in the lower extremities and is accompanied by slight rhythmical twitching. No changes in the peripheral reflexes accompany this seizure. The third type consists of sudden dilation of the pupils, absence of any reaction to light, opisthotonus, extension of all extremities, and flexion of the toes. This type of seizue was interpreted by Meduna and Gyarfas as a symptom of decortication.

Pollock,[3] Stein and Pollock,[4] Pollock *et al.*[5] demonstrated that carbon dioxide in certain concentrations inhibits the action of cortical convulsants.

Pollock and Bain[6] showed that changes in the metabolism of the brain produced by inhalation of carbon dioxide differ from those produced by cortical convulsants. In order to establish the anticonvulsant effect of carbon dioxide in man 92 experiments were carried out in 18 patients in the following way: First, the electro-shock dose of every patient was established, then carbon dioxide was adminis-

1 Waters, R. M., *New Orleans Med. and Surg. J.*, 1937, **219**, 90.

2 Meduna, L. J., and Gyarfas, K., Diseases of the Nervous System, in press.

3 Pollock, G. H., Thesis, Univ. of Illinois, College of Medicine, 1948.

4 Stein, S. N., and Pollock, G. H., PROC. SOC. EXP. BIOL. AND MED., in press.

5 Pollock, G. H., Stein, S. N., and Gyarfas, K., PROC. SOC. EXP. BIOL. AND MED., in press.

6 Pollock, G. H., and Bain, J. A., manuscript in preparation.

tered to the patients in sufficient concentration for a sufficient length of time to prevent the convulsant action of the current. The concentration of carbon dioxide varied from 15 to 30% and the duration of administration from 25 to 170 seconds. A description of the technic and of other details has been given by Pollock *et al.*[5]

Administration of carbon dioxide produced, in all our cases, the following convulsive phenomena: Initial periocular twitching followed by either an extensor hypertonus in all extremities or, more often, slight flexor spasm in the arms and extension in the legs, consecutive transitory plastic tonus, increasing occipital rigidity and opisthotonus, finally, a high degree of extensor rigidity appeared in all limbs, the fingers in *main d'accoucheur* position, toes in flexion. The dilated pupils reacted to light on all but 2 occasions. The tendon and skin reflexes could not be elicited on account of the muscular rigidity. Pathological reflexes were not seen during or after the convulsions though in some cases they preceded them. No tongue biting, incontinence, or postconvulsive stupor appeared. The respiratory and circulatory changes observed were those described by Meduna and Gyarfas.[2] The fits did not appear to be self-limiting. E.E.G., though obscured by muscular movements, was always clearly not of the grand mal type. The electrical activity of the cortex returned to normal shortly after discontinuing inhalation of carbon dioxide.

The clinical symptoms, the E.E.G., and the absence of postseizure stupor, correspond to decerebrate seizures. Our observations indicate, therefore, that the apparently different types of convulsive phenomena described by Meduna and Gyarfas[2] represent only different phases of one activity.

Summary. 1. Combination of E.S.T. with CO_2 inhibits the convulsion.

2. Inhalation of 30% CO_2 and 70% O_2 produced in all cases observed convulsive phenomena of decerebrate character.

16904

Mode of Action of Desoxypyridoxine.

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Antagonisms caused by metabolite analogues are generally accepted as being due to a competition between the analogue and the natural substrate for some enzyme system of the cell.[1] The inhibitor is thought to occupy a space on the surface of an enzyme which would normally be used by the metabolite. The relatively high ratios of analogue to metabolite usually required are considered as a reflection of relative affinity of the enzyme protein for the metabolite and its analogue. While these concepts have served as useful tools and have perhaps been entirely valid in many cases, it has also been apparent that they must be amplified to cover more complex situations; for example, where the substance used is a structural analogue of a vitamin which functions in the form of a coenzyme with a low dissociation constant. Such a case is offered by the action of desoxypyridoxine in competing with the vitamin B_6 group. This compound, 2,4-dimethyl-3 hydroxy-5 hydroxymethylpyridine, was reported by Ott[2] to act as a powerful antagonist of pyridoxine in the chick. Two moles of desoxypyridoxine counteracted 1 mole of pyridoxine when the latter was limiting. Emerson[3] showed that, in the rat, approximately 50 parts of desoxypyridoxine brought on the

1 Woolley, D. W., *Advances in Enzymology*, 1946, **6**, 129.

2 Ott, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 125.

3 Emerson, G. A., *Fed. Proc.*, 1947, **6**, 406.

signs of B_6 deficiency when a purified diet supplemented with pyridoxine was employed and that this ratio changed to 175 parts when a natural diet was fed. Additional studies by Mushett *et al.*,[4] Porter *et al.*[5] have demonstrated that desoxypyridoxine produces the pathological and biochemical symptoms associated with a vitamin B_6 deficiency so that there is no question that desoxypyridoxine antagonizes vitamin B_6. These studies revealed, however, that the antagonism was evident only when the vitamin B_6 supply was sub-optimal. With adequate vitamin B_6, desoxypyridoxine had little effect even at relatively high ratios. These conclusions are difficult to explain on a metabolite-analogue competition basis so that it was early realized that a more complex situation was present. Direct enzymatic studies were therefore undertaken to attempt to determine the mechanism of inhibition by desoxypyridoxine. The enzyme employed was tyrosine decarboxylase for which pyridoxal phosphate, a member of the vitamin B_6 group, is the coenzyme.[6] It was assumed that these studies would bear some relationship to the mode of action of desoxypyridoxine in the animal body but the concepts developed experimentally here may require some modification when applied to the complex situation in the animal.

The substrate competition concept is based upon the early studies of Quastel and Wooldridge[7] upon malonate inhibition of succinate transformations and this concept has largely colored the thinking of subsequent observers. However, when one is dealing with a vitamin analogue, the points of competition may be multiplied and a simple concept may require modification. The case of vitamin B_6 may be diagrammed as in Fig. 1. The known coenzyme form of the vitamin B_6 group is pyridoxal phosphate, yet in the diet pyridoxine, pyridoxal, and pyridoxamine are, within limits of stability and adsorption, essentially equally effective and have been shown to be converted into pyridoxal phosphate.[8] Desoxypyridoxine might act at points A, B, or C or it might be converted into desoxypyridoxine phosphate which could compete by uniting with the apo-tyrosine decarboxylase, or by displacing pyridoxal phosphate from the holoenzyme. Since the

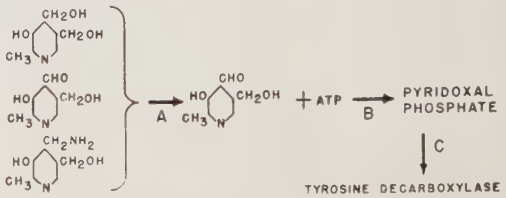

FIG. 1.
Reactions of members of the vitamin B_6 group.

analogue could act at one or at more than one of these points, it hardly seemed likely that a simple concept of substrate competition would suffice to describe the action of desoxypyridoxine. It therefore appeared worthwhile to attempt to distinguish experimentally between the possibilities mentioned particularly since enzymatic studies involving the possible interactions outlined above have been somewhat lacking.

Methods and materials. *Streptococcus faecalis,* strain R, was used as a source of tyrosine decarboxylase. For the preparation of the active (holo) enzyme the organism was grown at 25°C, 16-18 hours, in a medium composed of 1% each of tryptone, yeast extract, and glucose with 0.5% K_2HPO_4. The cells were harvested by centrifugation, washed with distilled water, and the vacuum dried preparation used as a source of the enzyme. The apoenzyme was prepared similarly using a medium deficient in the vitamin B_6 group.[9] The decarboxylation of tyrosine was measured at 37°C in 0.06 M acetate buffer at pH 5.5 as previously described.[6] Pyridoxal and desoxypyridoxine were used as their hydro-

[4] Mushett, C. W., Stebbins, R. B., and Barton, M. N., *Trans. N. Y. Acad. of Sciences,* 1947, **9**, 291.

[5] Porter, C. C., Clark, I., and Silber, R. H., *J. Biol. Chem.*, 1947, **167**, 573.

[6] Umbreit, W. W., Bellamy, W. D., and Gunsalus, I. C., *Arch. Biochem.*, 1945, **7**, 185.

[7] Quastel, J. H., and Wooldridge, W. R., *Biochem. J.*, 1927, **21**, 1224.

[8] Bellamy, W. D., Umbreit, W. W., and Gunsalus, I. C., *J. Biol. Chem.*, 1945, **160**, 461.

[9] Bellamy, W. D., and Gunsalus, I. C., *J. Bact.*, 1945, **50**, 95.

chlorides. Pyridoxal phosphate was standardized against a relatively pure sample[10] and was used as the calcium salt. Desoxypyridoxine phosphate will be described later. Adenosine triphosphate (ATP) was used as the sodium salt. Conventional Warburg technics were employed throughout.

Results. As outlined above, several possible sites of action of desoxypyridoxine are evident. These may be divided into two categories: (1) the inhibitions possible if the active agent is desoxypyridoxine, (2) the inhibitions possible if the active agent is desoxypyridoxine phosphate. While the second category proved to be correct, as will be shown later, it was still necessary to prove that there does not also exist an effect of desoxypyridoxine itself. This proof is provided in the first section. The tyrosine decarboxylase system employed did not include reactions A (Fig. 1) hence any effect of desoxypyridoxine (or its phosphate) on the conversion of pyridoxine or pyridoxamine to pyridoxal phophate could not be studied in this system.

Action of desoxypyridoxine. Since the results show that desoxypyridoxine as such has no effect upon the systems shown in Fig. 1, only a brief description of the experiments will be given. It was first necessary to test the possibility that desoxypyridoxine interfered with tyrosine decarboxylase presumably by displacing the pyridoxal phosphate. Beiler and Martin[11] have already reported that desoxypyridoxine was ineffective in this system up to concentrations as high as 300 γ per ml. In our hands concentrations as high as three times this level had no effect. Further, such concentrations had no effect upon the aspartic-glutamic transaminase from heart muscle[12,13] or upon the tryptophanase system of *E. coli*[14] both of which have somewhat higher dissociation constants than tyrosine decarboxylase and hence might be expected to show some degree of competition. Once pyridoxal phosphate is associated with the tyrosine decarboxylase protein, desoxypyridoxine does not displace it. Yet if the latter were allowed to reach the apoenzyme first it might prevent the combination of pyridoxal phosphate and apoenzyme. For this purpose a preparation was employed which at saturation had a Q_{CO_2} of 676. It responded in a linear manner to pyridoxal phosphate up to 8 mγ (calculated as the free acid) at which point the Q_{CO_2} was 460. It was reasoned that the ideal conditions for demonstrating any effect of desoxypyridoxine upon the combination of apoenzyme and coenzyme would be under conditions where the coenzyme was decidedly limiting since any competition would be immediately evident in a decreased rate of tyrosine decarboxylation. A point was therefore chosen approximately halfway up the region of linear response (3.8 mγ free acid) with the result (Table I) that desoxypyridoxine had no effect upon the combination of coenzyme and apoenzyme in spite of the fact that it was given adequate access to the enzyme before the pyridoxal phosphate was supplied. Similar results were obtained with a purified horse-heart transaminase preparation carried through the second precipitation in the procedure of O'Kane and Gunsalus[13] at which point it was 85% resolved.

Since from these data, desoxypyridoxine does not act on tyrosine decarboxylase (C, Fig. 1) the possibility remained that it acted by competing with pyridoxal for conversion to pyridoxal phosphate (point B, Fig. 1). It was reasoned that if ATP were made the limiting factor in the conversion of pyridoxal to pyridoxal phosphate, effects of replacing pyridoxal by desoxypyridoxine on the surface of the converting enzyme would be immediately evident. In this type of experiment a preparation was employed which had a very active converting system. Not very much is known about this enzyme and its occurrence in a given preparation is not exactly pre-

[10] Gunsalus, I. C., Umbreit, W. W., Bellamy, W. D., and Foust, C. E., *J. Biol. Chem.*, 1945, **161**, 743.

[11] Beiler, J. M., and Martin, G. J., *J. Biol. Chem.*, 1947, **169**, 345.

[12] Green, D. E., Leloir, L. F., and Nocito, V., *J. Biol. Chem.*, 1945, **161**, 559.

[13] O'Kane, D. E., and Gunsalus, I. C., *J. Biol. Chem.*, 1947, **170**, 425.

[14] Wood, W. A., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, 1947, **170**, 313.

TABLE I.
Lack of Effect of Desoxypyridoxine on Combination of Pyridoxal Phosphate and Apo-tyrosine Decarboxylase.

Pyridoxal phosphate, mγ	Ratio Desoxypyridoxine / Pyridoxal phosphate	Rate of tyrosine decarboxylation, Q_{CO_2}
0	0	61.6
1.92	0	158.8
3.84	0	242
3.84	3000	240
3.84	6000	246
7.68	0	448
11.52	0	540
15.36	0	576
19.20	0	676

Desoxypyridoxine, when supplied, incubated with apoenzyme 30 minutes before pyridoxal phosphate supplied.

TABLE II.
Effect of Desoxypyridoxine on the Synthesis of Pyridoxal Phosphate from Pyridoxal and ATP.

γ ATP*	Ratio Desoxypyridoxine / Pyridoxal	Q_{CO_2} on tyrosine
10	0	160
10	500	173
10	10,000	162
50	0	360
50	500	368
50	10,000	390
100	0	448
100	500	448
100	10,000	444

* $Na_4(ATP) \cdot 3H_2O$.

dictable. We have had preparations capable of converting 50% of the pyridoxal supplied to pyridoxal phosphate while others capable of converting only 1 to 2% or even less have been obtained under apparently the same conditions of cultivation and treatment of the organisms. However, in this case, a preparation actively converting 50% of the pyridoxal supplied was employed. ATP was made the limiting factor and two pyridoxal levels, one low (0.05 γ) and another relatively high (1 γ) were employed. The desoxypyridoxine was incubated with the enzyme for 30 minutes, to allow access to the enzyme, the pyridoxal and ATP were added simultaneously, incubated for 10 minutes to allow reaction B to proceed and the rate of tyrosine decarboxylation measured. The data for the lower level of pyridoxal are given in Table II; since the results with the higher level were similar they have been omitted. Within the limits of error of measurement there is no effect of desoxypyridoxine upon the formation of pyridoxal phosphate from ATP and pyridoxal. This is somewhat surprising since the competition theory as it is usually applied would predict that this reaction would be the site of action of desoxypyridoxine.

Action of desoxypyridoxine phosphate. The previous section has shown that desoxypyridoxine does not influence either the conversion of pyridoxal to its phosphate or the activity of the phosphate once formed. There remained, therefore, the possibility that desoxypyridoxine was not the active agent in the competition but that it was itself converted into desoxypyridoxine phosphate which was the competitive agent. Evidence bearing on this point was first sought in the conversion system (B, Fig. 1). The previous section has shown that when ATP was the limiting factor and adequate pyridoxal was available, desoxy-

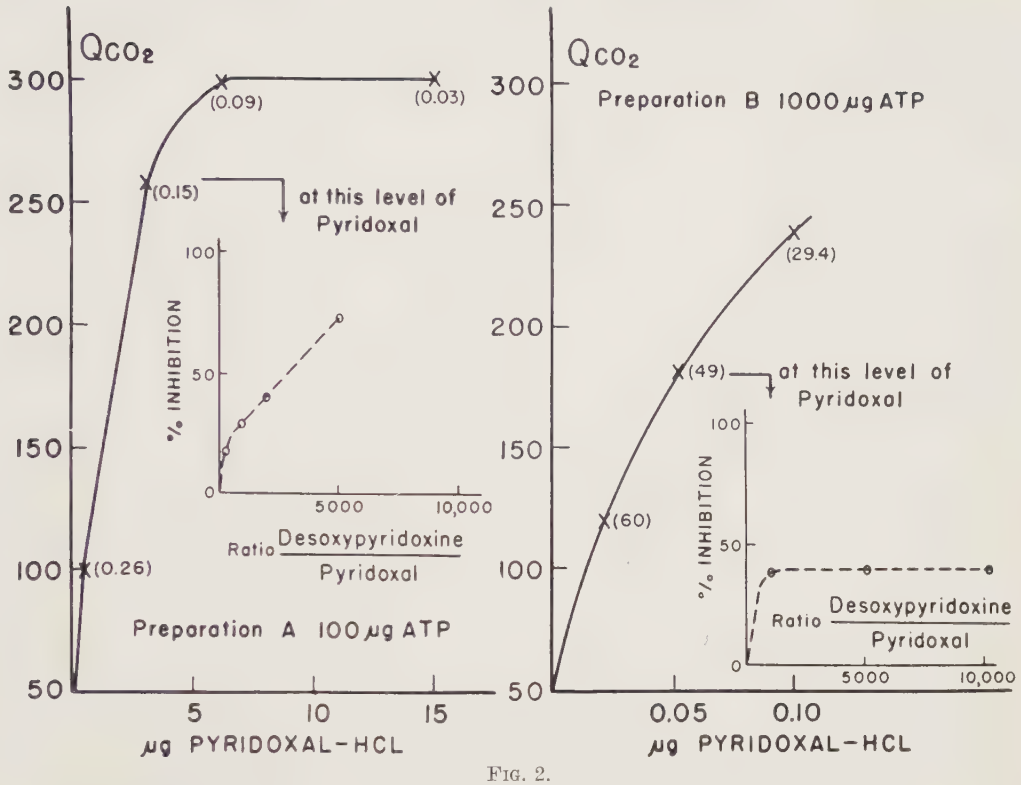

FIG. 2.

Enzymatic conversion of desoxypyridoxine to its phosphate and its interference with tyrosine decarboxylation.

In each case, 1 mg of the enzyme preparation was incubated with ATP and desoxypyridoxine for 15 min., pyridoxal added, in amounts indicated, incubated for 10 min., then tyrosine added and the rate of tyrosine decarboxylation determined. The solid lines show the rate of tyrosine decarboxylation at various levels of pyridoxal supplied in the absence of desoxypyridoxine and serve to show that pyridoxal is the limiting factor and that ATP is in excess. The values in parentheses give the percent of conversion of pyridoxal to pyridoxal phosphate. On the inset graphs the dashed curves show the extent of inhibition by various increments of desoxypyridoxine at the level of pyridoxal indicated.

pyridoxine could not compete to any detectable extent with pyridoxal. However, if pyridoxal were limiting so that some portion of the enzyme were free from pyridoxal, while ATP was at saturation or above, desoxypyridoxine might then become attached to the enzyme and be phosphorylated, the latter form interfering somehow with reaction C which would be inhibited. Data showing that such an inhibition does occur are given in Fig. 2.

Two types of preparations were used, one (preparation A) in which the conversion of pyridoxal to pyridoxal phosphate was relatively weak (0.2-0.3%) and another (preparation B) in which the conversion was relatively good (50-60%). The figures in parentheses on the graphs give the per cent of the pyridoxal supplied which appeared as pyridoxal phosphate (after a 10 minute incubation period). The pyridoxal phosphate was determined by comparing the activity found with that given by purified pyridoxal phosphate with the same preparation. As previously mentioned the conversion of pyridoxal to the phosphate by ATP is not a quantitative reaction and such preparations respond to pyridoxal phosphate in much lower quantities than to pyridoxal and ATP. For example, in the original enzyme preparation described[6] pyridoxal phosphate was 18-21 times as active as pyridoxal and ATP[10] indicating a conversion of approximately 5%. Different preparations of the dried cells of this organism

require different quantities of pyridoxal and ATP to reach either the maximum activity or the activity corresponding to a given level of pyridoxal phosphate. The cause of this difference is not known but it might possibly be related to different degrees of permeability of the cell to pyridoxal (thus preparation A might require 2 γ pyridoxal-hydrochloride to reach an internal concentration equivalent to that which preparation B was capable of reaching with only 0.06 γ). There are some indications that this is indeed the explanation but proof is lacking. However, by the use of two preparations of widely different "converting abilities" it was hoped to avoid complications arising from this effect.

The level of pyridoxal was chosen which gave good activity but which was below the saturation level. Desoxypyridoxine did, under these circumstances, show inhibitions as shown on the inserted graphs in each case. However, relatively high levels of desoxypyridoxine were required. Where the conversion to pyridoxal phosphate is relatively weak, desoxypyridoxine exerts its greatest effect. These data provide presumptive evidence that when ATP is in excess and pyridoxal limiting, there is some degree of inhibition by desoxypyridoxine. Since various other possibilities have been eliminated in the first section, the remaining possibility is that desoxypyridoxine is converted into desoxypyridoxine phosphate and that the phosphorylated derivative is the actual inhibitor.

Unfortunately desoxypyridoxine phosphate is not available nor is its preparation a matter of simple chemistry. The synthesis used by Beiler and Martin[11] is not a good one. Yields of pyridoxal phosphate using pyridoxal as the starting material are rarely more than 0.5% and there is no reason to expect any better yields with desoxypyridoxine. Somewhat improved syntheses have been obtained from pyridoxal by modifications of the original procedure[6] but rarely is more than a 5% conversion obtained. In the case of pyridoxal phosphate an assay method[6] is available which permits one to follow purification of this substance, but with desoxypyridoxine there is as yet no method of specifically estimating the phosphorylated form in a mixture of phosphorylated compounds. As a source of desoxypyridoxine phosphate we therefore employed a preparation* made by treating desoxypyridoxine with phosphoryl chloride, neutralizing the resulting mixture and separating the desoxypyridoxine phosphate as the calcium salt by alcohol precipitations. Such a material is not pure and may contain other substances. However, its organic phosphate content (4.52%) was assumed to be due to desoxypyridoxine phosphate and the amount added was calculated on this basis.

From the data cited previously, desoxypyridoxine phosphate interferes with reaction C (Fig. 1) in some manner. There are two parts to this reaction, one, a combination of the apoenzyme and pyridoxal phosphate, and two, the decarboxylation of tyrosine. The desoxypyridoxine phosphate does not measurably prevent the activity of tyrosine decarboxylase once the coenzyme is attached.

A vacuum dried preparation of *S. faecalis R* grown on a yeast extract medium had a Q_{CO_2} on tyrosine of 393. When 100,000 times as much desoxypyridoxine phosphate was supplied as the pyridoxal phosphate contained in the preparation a Q_{CO_2} of 362 was observed (8% inhibition); with 1 million times as much desoxypyridoxine phosphate the Q_{CO_2} was 372 (5% inhibition). These inhibitions are not significant. Beiler and Martin[11] have reported some degree of inhibition (approximately 30%) of tyrosine decarboxylase with a preparation of desoxypyridoxine phosphate. This type of inhibition has not occurred in our experiments.

Desoxypyridoxine phosphate does, however, interfere markedly with the combination of pyridoxal phosphate and apoenzyme. Data on this point are shown in Fig. 3 in which are plotted the results of a variety of conditions employed in determining the inhibitions at various ratios of desoxypyridoxine phosphate to pyridoxal phosphate. Curve A is the average of several types of experiments designed so that the desoxypyridoxine phosphate would have an equal or better chance of reaching the apoenzyme surface than the

* We are indebted to the Research Laboratories of Merck & Co., Inc., for this preparation.

pyridoxal phosphate. The individual treatments are plotted as discrete points on the curve. In one case desoxypyridoxine phosphate and pyridoxal phosphate were mixed and added to the preparation simultaneously on the assumption that this could provide an equal chance for both to reach the enzyme. They were then incubated for various periods up to 30 minutes before tyrosine decarboxylation was measured. In another treatment

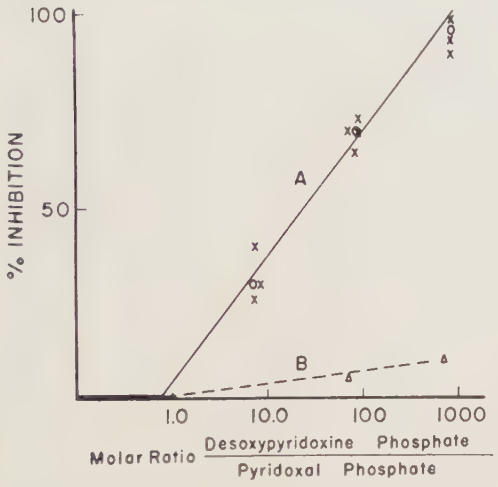

FIG. 3.

Competition between desoxypyridoxine phosphate and pyridoxal phosphate for tyrosine decarboxylase apoenzyme.

In curve A, desoxypyridoxine phosphate incubated with apoenzyme for 30 min. before pyridoxal phosphate added, or desoxypyridoxine phosphate and pyridoxal phosphate added simultaneously. After 10 min. of contact with pyridoxal phosphate, tyrosine was added and the rate of tyrosine decarboxylation determined. In curve B, the pyridoxal phosphate was added before the desoxypyridoxine phosphate. Pyridoxal phosphate used at level of 20 mγ (barium salt) per 3 ml, which is close to the top of the linear portion of the response curve of this preparation.

desoxypyridoxine phosphate was supplied first, incubated with the apoenzyme for 30 minutes, pyridoxal phosphate supplied, equilibrated for 10 minutes and tyrosine decarboxylation measured. Within the errors of measurement all of these combinations where desoxypyridoxine phosphate had an equal or better chance to reach the apoenzyme than pyridoxal phosphate, essentially the same degree of inhibition is observed. If, however, as shown in curve B, pyridoxal phosphate is supplied to the apoenzyme first and the combination between the two permitted, desoxypyridoxine phosphate supplied later has much less effect; in fact, the total inhibitions reached at the highest ratio was only 12%.

These data constitute evidence that the inhibition of tyrosine decarboxylase is due to the competition between desoxypyridoxine phosphate and pyridoxal phosphate for the apoenzyme. Pyridoxal phosphate has a greater affinity for this enzyme than desoxypyridoxine phosphate and if it is permitted to combine with the apoenzyme before desoxypyridoxine phosphate is present, the latter has but little effect.

A moment's consideration of the data presented will show that this locus of action of desoxypyridoxine is capable of explaining the results observed with desoxypyridoxine in growth studies. It is only when vitamin B_6 is deficient that there is any great opportunity for either the phosphorylation of desoxypyridoxine or its combination with apoenzymes and thus it is only under these circumstances that a marked inhibitory effect is observed. It is also evident that when one is dealing with a vitamin analogue the simple concept of substrate competition must be modified to include the other possibilities of competition. Further, there is no assurance that other analogues of vitamin B_6 might not act at different loci, hence any general viewpoint of metabolite analogue competition must be amplified. With such amplification it would seem reasonable that many of the present unexplainable phenomena of metabolite analogue competition might become understandable.

Summary. Upon the basis of the data presented, it is concluded that desoxypyridoxine exerts its inhibiting effect by being first converted into desoxypyridoxine phosphate which then competes with pyridoxal phosphate for the apoenzyme. This conclusion offers an explanation for the observation that in the animal, desoxypyridoxine exerts its antagonistic effect primarily under conditions of restricted vitamin B_6 intake.

16905

Effects of Cyanide Anoxia on Adrenal Gland of the Rat.

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Armstrong and Heim[1] reported that anoxic anoxia induces hypertrophy of the adrenal gland in rabbits. Since that time many papers have appeared on this subject (for references see Tepperman[2] and Nichols[3]). The most characteristic findings are hypertrophy of the gland and depletion of lipids from the cortex. To our knowledge the effects on the adrenal gland of other types of anoxia have not been studied, with the single exception of the work of Messerle[4] who made incidental observations during chronic exposure of pigeons to hydrogen cyanide gas. He reported gross hypertrophy of the gland but on histological observation found no cytological changes. It was thought that additional information on the mechanism of anoxic stimulation of the adrenal gland might be derived from experiments in which the oxygen tension of the arterial blood is normal.

Materials and methods. Twenty male rats of the Long-Evans strain, each weighing 150 g, were used in this study. Preliminary experiments indicated that 2 mg of sodium cyanide would kill a 150 g rat in about 30 minutes; accordingly, a maximum dose of 1 mg of cyanide was adopted for use in the remainder of the experiments. This was sufficient to give a severe but safe level of anoxia. Seven rats were injected subcutaneously with 1 mg of sodium cyanide contained in 1 ml of saline, followed by ½ mg of sodium cyanide every hour for the next 20 hours. Seven animals were injected with 1 mg of sodium cyanide followed by ½ mg of cyanide every half hour for the next 28 hours. The remaining 6 animals were treated as controls, being injected with 1 ml of saline every hour for 20 hours. All animals were killed by a blow on the head and the adrenals removed and placed in 10% formaldehyde for 24 hours. The surrounding fat was then carefully dissected away and the glands weighed. They were cut in half, embedded in gelatin and sectioned on the freezing microtome at 14 microns, stained and mounted as previously described.[3]

Results. The average weights of the adrenal glands were: controls 40 mg, treated 20 hours 45 mg, and treated 28 hours 54 mg. Histologically, the adrenals of the control animals were entirely normal as compared with other normal glands studied in this laboratory (Fig. 1). The glands of the animals treated for 20 hours showed marked depletion of lipids in the inner cortical zones (fasciculata and reticularis), while the zona glomerulosa remained relatively unchanged. The animals treated for 28 hours showed the same adrenal changes except to a more striking degree (Fig. 2); in certain of these animals the lipids were depleted in the zona glomerulosa to almost the same extent as in the inner zones. These histological changes are the same as those that occur in anoxic anoxia.

Discussion. As previously mentioned Messerle[4] reported gross hypertrophy of the adrenal, but no cytological changes, on chronic exposure of pigeons to hydrogen cyanide; however, he used standard hematoxylin and eosin preparations which had been dehydrated in alcohols that would dissolve out the fats.

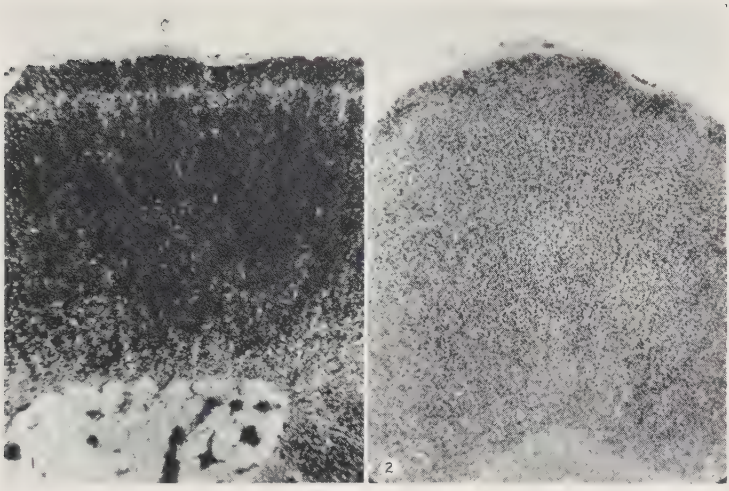
Adrenal cortical lipid depletion in anoxia is believed to be mediated by way of the pituitary gland since the zones first involved (*i.e., fasciculata* and *reticularis*) are known to be under control of the pituitary; these zones elaborate the fraction of the cortical hormone which influences carbohydrate meta-

1 Armstrong, H. G., and Heim, J. W., *J. Aviation Med.*, 1938, **85**, 162.

2 Tepperman, J., Tepperman, H. M., Patton, B. W., and Nims, L. F., *Endocrinology*, 1947, **41**, 356.

3 Nichols, J., *J. Aviation Med.*, 1948, **19**, 171.

4 Messerle, N., *Virchow's Arch.*, 1926, **262**, 305.



Frozen sections stained with Sudan III. Photographed with a green Wratten filter No. 29 at 135 diameters magnification and reduced to 68 by the engraver.

FIG. 1. Cortex of the adrenal gland of a normal animal.

FIG. 2. Cortex of the adrenal gland of an animal injected with 1 mg of sodium cyanide followed by ½ mg every half hour for 28 hours. Note depletion of lipids especially in the inner zones while the outer zona glomerulosa is relatively resistant.

bolism. Alterations of carbohydrate metabolism may be prevented by ablation of either the pituitary or adrenal (Evans[5]). The zona glomerulosa, which reacts last to anoxia, is not under control of the pituitary and, at least in the rat, regulates electrolyte balance (Nichols[6]; Deane *et al.*[7]).

The factor(s) responsible for pituitary stimulation in anoxia are uncertain. In anoxic anoxia, the most likely factors are the reduced oxygen tension of the arterial blood and the hypocapnia incident to hyperventilation. That hypocapnia may be a significant factor is indicated by the observation of Hailman[8] and that of Langley *et al.*[9] that the addition of sufficient carbon dioxide to give a partial pressure of 30 to 48 mm Hg prevents the usual anoxic changes in the adrenal gland and also prevents the increased deposition of liver glycogen. The changes in the adrenal are probably the result of the lowered blood carbon dioxide tension *per se*, rather than the resultant disturbance in acid base equilibrium. In unpublished experiments in this laboratory it was found that alkalosis and acidosis induced by oral administration of sodium bicarbonate and ammonium chloride respectively had no effect on adrenal changes in anoxic anoxia.

In this study we have not eliminated the hyperventilation possibility, since the animals did hyperventilate after each injection. In order to avoid this it would be necessary to denervate the carotid body. However, our findings indicate that histotoxic anoxia induced by sodium cyanide produces the same changes in the adrenal as does anoxic anoxia and that these changes can occur while the arterial blood is saturated with oxygen.

Summary. Sodium cyanide was administered in small repeated sublethal doses to rats. The resulting changes in the adrenal gland were hypertrophy and marked depletion of lipids in the inner zones of the cortex. This effect is identical with that of anoxic anoxia and occurs while the arterial oxygen tension is normal.

5 Evans, G., *Am. J. Physiol.*, 1935, **114**, 297.

6 Nichols, J., *Arch. Path.*, 1948, **45**, 717.

7 Deane, H. W., Shaw, J. H., and Greep, R. O., *Endocrinology*, 1948, **43**, 133.

8 Hailman, H. F., *Endocrinology*, 1944, **34**, 187.

9 Langley, L. L., Nims, L. F., Harvey, T. S., and Clarke, R. W., *N.R.C. Committee on Aviation* Medicine Report No. 108, 1943.

16906

Estrogen Response and Pigmentation of the Uterus in Vit. E-Deficient Rat.*

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(With the technical assistance of R. E. Johnson and A. Fuhr.)
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Several investigations have been made concerning the possibility that a relationship exists between the biological action of estrogen and vitamin E.[1-3] Although some of these experiments have suggested an "activating" effect of tocopherol on estrogen, the data have not been conclusive. A related problem, that of the role of the ovaries in the development of the characteristic pigmentation of the uterus during vitamin E deficiency, also remains unsettled. Therefore the present experiments were designed to determine the effect of alpha-tocopherol deficiency on the uterine response to estrogen and the effect of ovariectomy on the development of uterine pigmentation in vitamin E-deficient rats.

Materials. The animals used in the present experiments were obtained in the following manner. Female rats of the "Sherman" strain were reared from weaning on Rockland rat diet. Upon reaching 2-3 months of age they were placed on a vitamin E-deficient diet (Table I) which limited the daily intake of alpha tocopherol to approximately 30 μg per rat.† Within one week after they had been placed on the deficient diet, the animals were mated with non-deficient males. The tocopherol-deficient offspring were used in the present experiments as indicated below. After weaning of the young, the mothers were returned to the Rockland diet for 3 weeks and the same breeding procedure was then repeated.

Within 3 days after birth the young were pooled and 6 females were given to each of several mothers. These young were thereafter considered as "litter-mates". Between 3 and 4 weeks of age they were weaned and ovariectomized. Thirty-nine animals were continued on the vitamin E-deficient diet and 48 were placed on the same ration supplemented with 3 mg of synthetic dl alpha-tocopherol acetate per 100 g diet.‡ Eight unspayed animals were kept on the deficient diet as controls.

The uterine response to estrogen was determined with crystalline alpha-estradiol benzoate as indicated below.§ The hormone was dissolved in sesame oil which was freed of tocopherol by previous treatment with ferric chloride.

For histological examination the uteri were fixed in Bouin's fluid within 10 minutes after the animals were killed. Tissue specimens were dehydrated in ethyl alcohol, embedded in paraffin and sections were cut 7 microns in thickness. Parallel sections were stained with hematoxylin and eosin and with carbol-fuchsin to determine the presence and dis-

* Aided by a grant from the Williams-Waterman Fund of the Research Corporation.

1 Spoto, Pompeo, *Z. Vitaminfschg.*, 1940, **10**, 235.

2 Beerstecher, E., Jr., *Endocrinology*, 1941, **28**, 344.

3 Tobin, Charles E., and Birnbaum, Jean P., *Arch. Pathol.*, 1947, **44**, 269.

† About one-half of the determinations of the tocopherol content of the basic diet were carried out according to the method of Kaunitz and Beaver.[6] For the remaining tests we are greatly indebted to Drs. Philip L. Harris and Mary L. Quaife of the Distillation Products, Inc.

6 Kaunitz, H., and Beaver, J. J., *J. Biol. Chem.*, 1944, **156**, 653.

‡ The alpha tocopherol acetate and the other synthetic vitamins were supplied through the courtesy of Dr. Leo A. Pirk of Hoffmann-LaRoche, Inc.

§ The alpha estradiol benzoate was supplied through the courtesy of Dr. Kenneth W. Thompson of Roche-Organon, Inc.

TABLE I.
Composition of the Tocopherol-deficient Diet Used.

Basal mixture	%	Supplements to basal mixture	mg/kg
Lard	10	Thiamine chloride	2
Casein (Borden's crude) No. 453	30	Riboflavin	4
Cerelose	54	Pyridoxine	4
Celluration	2	Nicotinic acid	100
Salt mixture (Hawk Oser)	4	Choline	1000
		Vit. K	4
		Para-amino-benzoic acid	300
		Ca pantothenate	10
		Percomorphum oil (ml/kg)	0.2

tribution of acidfast pigment.

Experiments and observations. 1. Uterine response to estrogen. The uterine weights of deficient and non-deficient ovariectomized animals after the administration of alpha-estradiol benzoate are given in Fig. 1. In

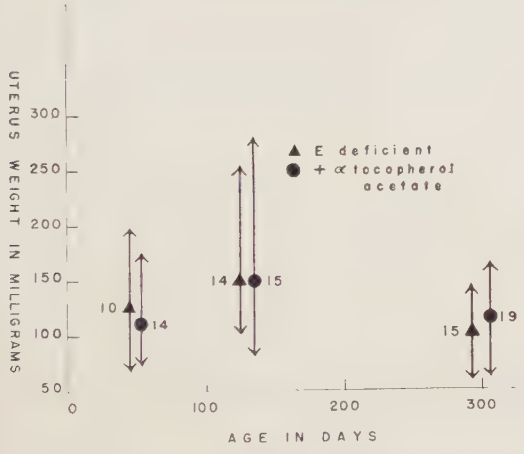

FIG. 1.

Effect of alpha estradiol benzoate upon the uterine weight of spayed rats on a vitamin E-deficient and a tocopherol-supplemented purified diet. The triangles and circles indicate average uterine weights; the figures adjacent to the triangles and circles indicate the number of animals used in the respective series.

the age group of 40-50 days, 1 μg of hormone dissolved in 0.2 cc of sesame oil was injected subcutaneously 48 hours before sacrifice. In the groups 120-137 and 280-309 days of age, a total of 4 μg of the estrogen, divided in successive daily doses of 1 μg in 0.2 cc of oil, was injected. The final dose was given 48 hours before sacrifice. Several of the deficient and tocopherol-supplemented animals in the 120 day group had received a total of 11 μg of estradiol benzoate between the 43rd and 78th day. Since their uterine response was similar to that of the other animals in this age group, they have been included in the final observations.

There is no significant difference in the uterine response to estrogen between the tocopherol deficient and tocopherol-supplemented animals in the same age group. There is, however, a difference in the response between animals in the 120-137 day and the 280-309 day groups, irrespective to tocopherol administration. Statistical evaluation of the mean uterine weights of these two groups yielded a probability factor of 3.7 indicating that the response of the 4-month-old rat to estrogen is significantly greater than that of the 9-10-month-old animal.

2. Uterine pigmentation. As reported by previous investigators, a brownish pigmentation of the uterus was macroscopically discernible in the non-spayed rats maintained on the tocopherol-deficient diet for approximately 300 days. In sharp contrast, there was no evidence of pigment deposition in the majority of the spayed animals fed the same diet for the same period of time. As would be expected, no pigmentation occurred in either spayed or non-spayed animals receiving the tocopherol-supplemented diet.

Histological examination of the uteri of 8 unspayed deficient animals revealed that the muscle cells of the myometrium were filled with numerous uniformly small acid-fast pigment granules. In addition, a considerable number of pigment-containing cells were scattered throughout the intermuscular connective tissue and, to a lesser extent, the endometrial stroma. These cells, presumably macrophages, varied in size and shape, but

were generally irregularly rounded. The larger were often multinucleate. Unlike in the myometrium, the pigment inclusions in these connective tissue cells varied considerably in size. No pigment was present in the surface and glandular epithelium of the endometrium. These findings are in good agreement with previous histological studies of the uterus in the vitamin E-deficient rat.

The uteri from 8 of 9 spayed animals maintained on the tocopherol-deficient diet exhibited little or no pigmentation of the myometrial cells and contained but few macrophages with pigment inclusions. The remaining animal in the group resembled the unspayed deficient animals in amount and distribution of pigmentation. Neither the unspayed nor spayed animals maintained on the tocopherol-supplemented diet showed microscopic evidence of abnormal uterine pigmentation.

Discussion. The present observations clearly indicate the uterine response to estrogen in vitamin E-deficient ovariectomized rats does not differ significantly from that of tocopherol-supplemented controls. This finding suggests that an intimate physiological relationship between the two substances is not very probable. It is possible, however, that conditions in the unovariectomized animal may be materially different in regard to the metabolism of estrogen and tocopherol.

Studies concerning the influence of vitamin E deficiency upon uterine pigmentation have been made by Mason and Emmel.[4] Unlike the present experiments, these authors did not find unequivocable evidence concerning the influence of ovariectomy upon the development of uterine pigment in their vitamin E-deficient rats. These contrary results may have been caused by the different experimental diets used.‖ The fat content of the diet used by Mason and Emmel was double that of our diet and, in addition, their diet contained 2% cod liver oil. This is of particular significance since it has been shown that pigmentation in vitamin E-deficiency is intimately related to the intake of fat, especially if it contains large amounts of highly unsaturated compounds as does cod liver oil.[5] However, the apparent non-essentiality of the ovary in uterine pigment deposition in rats maintained on a high fat intake as compared with rats on low fat intake must remain unexplained at the present time.

The question may arise as to whether the absence of pigment in our vitamin E-deficient spayed animals might be due to the estrogen treatment just preceding sacrifice. This is not the case since, in other work not reported here, tocopherol-deficient spayed animals which had not received estrogen exhibited no macroscopic evidence of uterine pigmentation.

Summary. (1) Rats ovariectomized at weaning were maintained on a vitamin E-deficient diet for from 6 weeks to 10 months. No difference in the uterine weight response to injected estradiol benzoate was discernible between these animals and control animals of the same age maintained on the same diet supplemented by alpha-tocopherol acetate

(2) The uterine weight response to alpha-estradiol benzoate was significantly greater at 3 months of age than at 10 months regardless of tocopherol administration.

(3) Accumulation of acid-fast pigment in the uterus is characteristic of intact rats maintained on a vitamin E-deficient diet. Ovariectomy at weaning prevented the appearance of uterine pigmentation in animals maintained on the deficient diet for as long as 10 months.

[4] Mason, Karl E., Dam, Henrik, and Granados, Humberto, *Anat. Rec.*, 1946, **94**, 265.

‖ It has been suggested by Dr. Karl E. Mason that the differences in the results may have been due partly to their use of Zenker's fixative whereas Bouin's fixative has been used in the present studies. For this and many other creative criticisms we are highly indebted to Dr. Mason.

[5] Mason, Karl E., and Emmel, Anna F., *Anat. Rec.*, 1945, **92**, 1945.

16907 P

Prevention of Liver Necrosis following Ligation of Hepatic Artery.

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From the Department of Physiology, University of Toronto.

In dogs, ligation of the hepatic artery is followed in approximately 100% of cases by death, as a result of a peculiar necrosis of the liver. In a series of experiments extending over many years we have tried to prevent this mishap by arterializing the blood flow of the portal vein. This year we appeared to be unusually successful, in that the animals survived such ligation. Examination at autopsy, however, always disclosed that the arterioportal anastomosis was thrombosed, although the liver was grossly normal. During this year penicillin has been administered following the operations in one series of animals. Another series did not receive penicillin. The wholly unexpected finding was made, that when dogs with totally ligated hepatic arteries are given massive doses of penicillin, intraperitoneally and intramuscularly for one week, they usually survive, whether or not the gall bladder has been removed. The latter becomes gangrenous, often ruptures, but even this occurrence is well tolerated by the animal unless generalized bile peritonitis develops.

It is suggested that the immediate life-saving function of the hepatic artery is to maintain oxygen tension at a level incompatible with the proliferation of anaerobes constantly present in hepatic tissue.

16908

Relationship of Blood Sugar and Hypoproteinemia to Antibody Response in Diabetics.*

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(Introduced by E. Spiegel.)
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Evidence has been presented that protein depleted animals as compared with normal controls possess little ability to manufacture antibodies. The poor antibody response in animals can be restored to normal by protein repletion.[1-3] We have reported on similar studies in man, particularly in the hypoproteinemic patient as seen in a large general hospital.[4]

The purpose of this paper is to direct attention to the fact that the diabetic with such complications as gangrene, osteomyelitis, etc., has a diminished antibody production; this low antibody response to antigenic stimulation appears to bear relationship to the blood protein values rather than to the fasting blood sugar levels.

For this study a representative group of 64 diabetic patients attending the metabolic

* Grateful acknowledgement is made of encouragement and supply of material by Dr. Gustav Martin and Mr. Steven Horochak of the Medical Research Department of the National Drug Company.

1 Cannon, P. R., *J. Immunol.*, 1942, **44**, 107.

2 Cannon, P. R., Chase, W. E., and Wissler, R. W., *J. Immunol.*, 1943, **47**, 133.

3 Wissler, R. T., Woolridge, R. L., Steffee, C. H., Jr., and Cannon, P. R., *J. Immunol.*, 1946, **52**, 267.

4 Wohl, M. G., Reinhold, J. G., Rose, S. B., Adams, L. A., Harvey, T., Francis, M., and Clough, G., *Arch. Int. Med.*, in press.

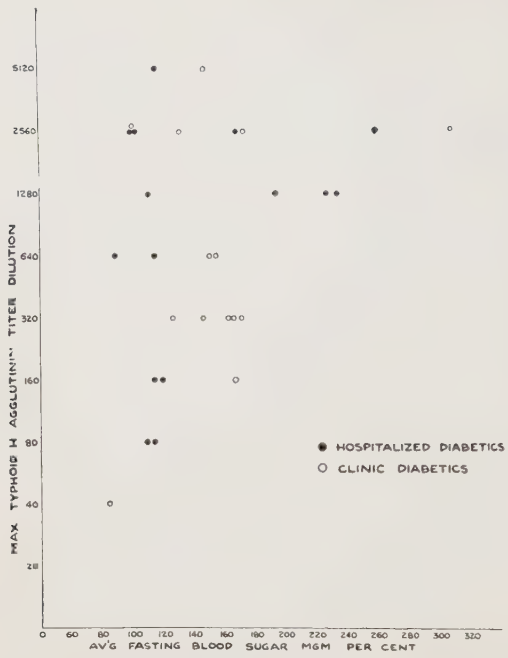

CHART 1.

Relation of the fasting venous blood sugar to antibody response.

clinic and housed in the metabolic wards of the Philadelphia General Hospital were selected. Eleven nondiabetic persons served as controls. Nineteen hypoproteinemic patients received protein supplementation in the form of lactalbumin hydrolysate or casein concentrate. The diet of the patients in the supplemented groups remained isocaloric with that of the nonsupplemented persons by reducing the fat intake by grams equivalent to the calories supplied by the additional protein. The average diet of diabetic men contained 2100 calories, of women 1800 calories.

The patients were studied chemically and immunologically by the methods previously described.[4] In addition, in 14 patients nitrogen balance studies were performed for a period of 36 to 55 days; detailed studies of the latter will be reported elsewhere. The data are plotted in Charts 1 and 2.

In Chart I the ordinates indicate the peak agglutination titre to typhoid H antigen; the abscissas indicate the corresponding average fasting blood sugar levels in mg %. Study of Chart I reveals that there is no relationship between the fasting blood sugar levels and the serum agglutinin content. To illustrate the patients with the highest titres (1:1280 to 1:5120) have fasting venous sugars from 100 to 300 mg %. On the other hand, titre levels of 1:160 or less were found in patients with average fasting venous blood sugars of 170 mg % or less. It would appear then that patients with hyperglycemia do not demonstrate a decreased capacity to produce antibody.

An analysis of Chart 2 shows that the diabetic patients with hypoproteinemia (serum albumin levels below 4 g %, the lower limit of normal for the method used) showed a lower average agglutination titre than the diabetic patients with normal blood protein values. The average serum albumin of the normal controls was 4.87 g %, of the clinic diabetic groups 5.27 and 4.28 g % respectively, of the 2 hospitalized diabetic groups

Typhoid H Agglutinin Titer in Diabetes Mellitus.

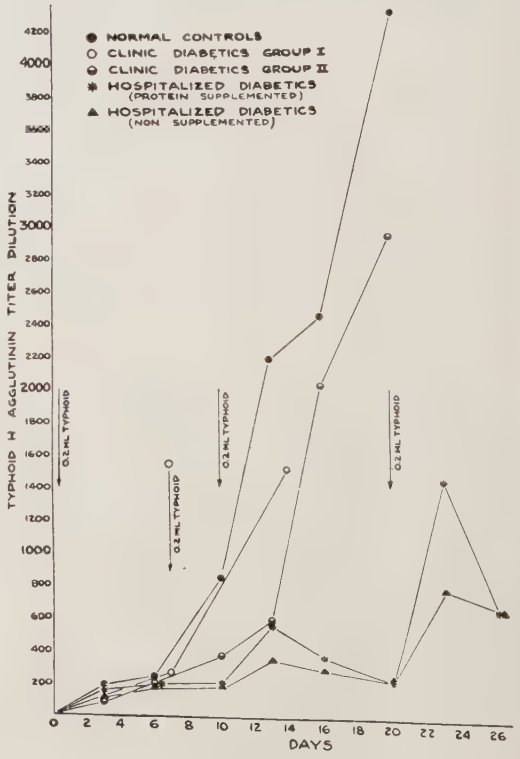

CHART 2.

Comparison of antibody formation in normal controls and diabetic adults with special reference to normoproteinemic and hypoproteinemic diabetics.

TABLE I.
Relation of Protein Repletion (Positive N Balance) to Antibody Titre in Diabetics.

	Balance				
	Days	Avg g N/DA.	Max. titre	Avg alb.	Supplemented
			1:		
Ha.	20	7.5	40*	3.8	+
Ab.	36	6.6	640	4.7	+
We.	8	5.4	1280*	4.7	+
Cr.	36	4.9	320*	4.4	+
Pe.	74	4.7	5120	3.4	—
Sm.	62	4.4	320	3.1	+
Wat.	42	4.2	640	4.3	+
Cu.	55	2.6	2560	4.4	—
War.	49	—0.3	160	4.0	—

* Two injections typhoid.

TABLE II.
Average Serum Albumin Concentration.

Group	Day 0	17	27
Normal	4.87	4.85	4.80
Clinic diabetics			
I	5.27	5.48	5.37
II	4.28	4.18	4.40
Hospitalized diabetics			
(Supplemented)	3.11	3.77	3.87
(Non-supplemented)	3.31	3.41	3.41

3.11 and 3.31%. (See Table II). These figures remained essentially unchanged during the study except for the protein supplemented group whose albumin rose from a mean of 3.11 g to 3.87 g %.

The average highest titre of 16 diabetic patients with hypoproteinemia but nonsupplemented was 1:800 as compared with the average highest titre of 1:2900 in 9 normoproteinemic uncomplicated diabetics who only received 2 typhoid injections, whereas the hypoproteinemic group received 3 injections. There were 20 normoproteinemic clinic diabetics who also received 2 typhoid injections and whose highest titre was 1:1500 seven days after the second injection.

Nineteen hypoproteinemic patients were provided with oral supplements of protein in the form of lactalbumin hydrolysate or casein concentrate. The diets were carefully calculated and sufficient supplementary protein was added to double the daily protein intake as compared with that of the other hypoproteinemic group.

The results of this study showed an improvement in antibody response. During the first 10 days after initial injection the average titre in both supplemented and unsupplemented groups was approximately the same. Following each subsequent typhoid injection there was a greater rise in titre in the supplemented as compared with the nonsupplemented groups.

It is to be noted, however, that the best antibody response was obtained in the outpatient normoproteinemic diabetics (who were not suffering from an active complication of the disease) even though they received no protein supplementation.

The addition of extra protein to the diet did not appear to result in an increase in the severity of the diabetes during the experimental period of 30 days. There was no appreciable change in blood sugar levels, nor was there any change in plasma CO_2 combining power nor any increase in insulin requirements. It is of interest that there was no apparent relation between the degree of nitrogen storage and the peak of typhoid agglutinin titres (Table I), indicating that the retention of protein (as N) was not immediately reflected in an increase of antibody formation.

Whether the enhanced antibody-producing capacity is due specifically to an increase in the body's protein "pool" for globulin-antibody production or some other factor needs further study.

Summary. Sixty-four diabetic patients were studied immunologically and chemically. Nineteen diabetic patients with hypoproteinemia received nitrogen supplementation in the form of lactalbumin hydrolysate or casein concentrate. The following conclusions appear to be warranted: 1. The decreased capacity to produce antibody in the diabetic is apparently not related to hyperglycemia. 2. Diabetic patients with hypoproteinemia showed a lower average agglutination titre than those with normal blood protein values. 3. Oral supplementation with lactalbumin hydrolysate or casein concentrate enhanced antibody formation.

We are indebted to Drs. Edward S. Dillon, Anthony J. Sindoni, Jr., John G. Reinhold and Henry P. Schwarz for their suggestions and cooperation.

16909

Streptocin, Antibiotic Isolated from Mycelium of *Streptomyces griseus*, Active Against *Trichomonas vaginalis*, and Certain Bacteria.*

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Schatz and Waksman[1] first reported that *Streptomyces griseus* produces, in addition to streptomycin, another antimicrobial substance. The latter was found to a small extent in the culture fluid but was present to a large extent in the mycelium, from which it was extracted with ether and chloroform. This crude substance was more active against the avian strain of *Mycobacterium tuberculosis* than against the human strain, the reverse being true for streptomycin. Further studies[2] showed that the material was soluble in various organic solvents, and was active largely against certain gram-positive bacteria, and only to a limited extent against the gram-negative organisms; it was later found to be active also against fungi, including both parasitic and saprophytic forms. One of the fractions of a similar extract isolated from a different strain of *S. griseus* was found to be trichomonadicidal. This substance can be designated as *streptocin*.

Whiffen, Bohonos and Emerson[3] reported the isolation from the culture medium of *S. griseus* of an antibiotic substance which is active against yeasts and fungi, and which is not active against bacteria. This antibiotic, designated as actidione, was soluble in organic solvents and in water, and was thermostable; it is distinctly different, however, from the trichomonadicidal substance which is reported here.

Isolation and antibacterial properties of crude preparation. Ether extracts obtained from the culture filtrate or from the mycelium of *S. griseus* gave the same antimicrobial spectra. The yields varied from 35 to 55 mg per liter of culture filtrate, and from 55 to 75 mg for the mycelium produced in one liter of medium. There was considerable variation, however, in the potency of the crude preparations depending on the strain of the organisms and on the conditions of growth. Much smaller amounts of the crude preparation were obtained from the submerged mycelium of two streptomycin-producing strains of *S. griseus* than from stationary pellicles, as shown in Table I. The stationary preparations were also more active than those obtained from the submerged mycelium (Table II).

By the turbidimetric method, it was established that 60 μg of the crude substance was sufficient to inhibit the growth of the pathogenic *M. tuberculosis* H37, including both the streptomycin-sensitive and resistant strains (Table III).

Isolation and purification of streptocin for trichomonadostatic and trichomonadicidal studies. For trichomonadostatic studies, a strain of *Trichomonas vaginalis* (No. 2) was used;[4] the organism was grown on a simplified trypticase-serum medium.[5] The method of inoculation and growth and the procedure employed in measuring growth are described

* Journal Series paper, New Jersey Agricultural Experiment Station, Rutgers University; The State University of New Jersey, Department of Microbiology.

1 Schatz, A., and Waksman, S. A., Proc. Soc. Exp. Biol. and Med., 1944, **57**, 244.

2 Waksman, S. A., Schatz, A., and Reilly, H. C., *J. Bact.*, 1946, **51**, 753.

3 Whiffen, A. J., Bohonos, N., and Emerson, R. L., *J. Bact.*, 1946, **52**, 640; Leach, B. E., and Ford, J. H., *J. Am. Chem. Soc.*, 1948, **70**, 1223; Whiffen, A. J., *J. Bact.*, 1948, **56**, 283.

4 Johnson, G., Trussell, M., and Jahn, F., *Science*, 1945, **102**, 126.

5 Kupferberg, A. B., Johnson, G., and Sprince, H., Proc. Soc. Exp. Biol. and Med., 1948, **67**, 304.

TABLE I.
Production of Ether-soluble Antimicrobial Substances by 2 Strains of *S. griseus* Grown in Stationary and Submerged Cultures.

Strain of *S. griseus*	Culture	Yield,* mg	Activity, in dilution units, per 1 mg		
			S. aureus	*B. subtilis*	*B. mycoides*
3498	Stationary	131	65	114	114
3498	Submerged	31	9	16	16
3463-4	Stationary	318	27	47	47
3463-4	Submerged	38	13	13	13

* Per 5 liters of medium.

TABLE II.
Antimicrobial Activity of Ether-soluble Extract of *S. griseus*.
Dilution units per 1 mg of crude extract.

Organism	Activity
Staphylococcus aureus	120
Bacillus subtilis	120
B. mycoides	120
Mycobacterium phlei	15
Mycobacterium 607	4
M. tuberculosis H37Rv	2.4
M. tuberculosis H37RvR	2.4
M. avium	10.7
Escherichia coli	1
Proteus vulgaris	1
Serratia marcescens	1
Candida albicans	1
Trichophyton mentagrophytes	1.4

TABLE III.
Bacteriostatic Activity of Ether Extracts of *S. griseus* on *M. tuberculosis var. hominis*.

	Days of incubation			
	4	7	11	23
μg/ml of medium	Turbidimetric readings (in logs)			
Streptomycin-sensitive strain H37Rv				
200	—*	—	—	—
100	—*	—	—	—
60	—*	—	—	—
20	0	0	2	14
10	0	3	11	90
6	0	5	14	83
2	0	4	14	90
0	1	5	17	80
Streptomycin-resistant strain H37RvR				
200	—*	—	—	—
100	—*	—	—	—
60	—*	—	—	—
20	0	0	2	14
10	0	0	5	20
6	0	2	6	25
2	0	0	4	20
0	0	3	7	25

* A heavy precipitate formed but no growth occurred, as determined by plating procedures.

elsewhere.[6] To prevent bacterial contamination, 250 units per ml of medium of both streptomycin[7] and penicillin[4] were added to the suspension of the trichomonads, since these antibiotics had no effect either upon cell multiplication of the trichomonads or upon the activity of streptocin.

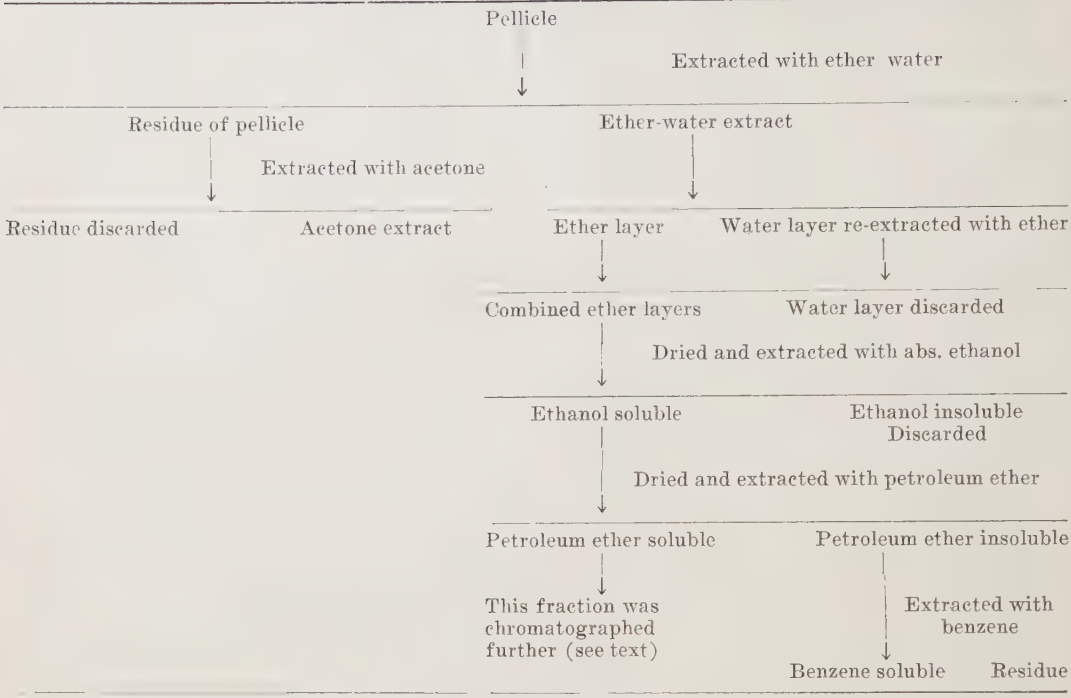
The streptocin used in the preliminary trichomonadostatic and trichomonadicidal studies was first isolated from a strain of *S. griseus* (No. 3533) which produced antibiotics active largely against gram-positive bacteria and trichomonads and was, therefore, neither streptomycin nor grisein. The culture was grown on a glucose-peptone-meat extract-NaCl-tap water medium in static or shaken condition. The mycelial growth was extracted in a Soxhlet with redistilled diethyl ether, some distilled water being added twice daily to the extraction chamber. The extraction was continued 24 hours after the ether became colorless. The ether-water mixture was removed, and redistilled acetone used for further extraction of the residual mycelium. This extraction was carried out as above. Only the fractionation of the ether-water extract is discussed in this paper.

The ether and water layers were separated, and the latter was reextracted with ether. The water then contained no trichomonadicidal activity and was discarded. The two ether fractions were combined, and the ether was removed under vacuum. The brown, oily

6 Johnson, G., and Kupferberg, A. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 390.

7 Trussell, R. E., *Trichomonas vaginalis and Trichomoniasis*. C. C. Thomas, Springfield, Ill., 1947, 61.

FIG. 1.
Extraction and Fractionation of Pellicle of *S. griseus* 3533.



residue was extracted with absolute ethanol. The ethanol-insoluble material was found to have no activity. The ethanol solution was distilled *in vacuo* and the resulting dry, yellow solid extracted with petroleum ether (b.p. 39-42°). The solvent was removed and the material dried over calcium chloride. The petroleum-ether-insoluble material was extracted with benzene; the benzene-soluble and -insoluble portions were dried *in vacuo*. The various steps used in this procedure are illustrated in Fig. 1.

The 3 fractions were chromatographed on columns of Brockman's Alumina (Merck). Since the general nature of the eluants of these columns was similar, only one of these, the petroleum-ether-soluble material, is discussed. This material was redissolved in dry petroleum ether and placed on the column in this solvent. The chromatogram was developed with increasing concentrations of benzene up to the pure solvent and then successively with acetone, ethanol, and methanol, until a 50-50 mixture of methanol-ethanol was reached. The material eluted at this point yielded, on evaporation of the solvent, an apparently crystalline material which possessed trichomonadicidal activity. Increasing concentrations of methanol were then employed for further development of the chromatogram. When 1% glacial acetic acid in methanol was used, more material was eluted, which on evaporation of the solvent, yielded a needle-like crystalline material which appeared in the form of rosettes. This preparation of streptocin had a high *in vitro* trichomonadicidal activity; its antibacterial spectrum is shown in Table IV. In contrast to the crude mixture isolated from the streptomycin-

TABLE IV.
Antimicrobial Activity of Crystalline Streptocin
Dilution Units per 1 mg of Streptocin.

Organism	Activity
E. coli	<3
B. mycoides	3
S. aureus	3
B. subtilis	75
M. ranae	<8
Mycobacterium 607	13
Mycobacterium 607R	13
M. avium	8

TABLE V.
Comparative Effects of Crystalline Streptocin and Actidione on Various Trichomonads.
Effects of 50 μg/ml of antibiotics upon the trichomonad population after 48 hours incubation at 37°C, numbers* expressed as cells/mm³.

Antibiotic	*T. foetus Br.*	*T. gallinae*	*T. vaginalis*
None	5,140	1,460	1,600
Streptocin	182	1,620	0
Actidione	1,490	0	10

* These figures represent the average of duplicate determinations.

TABLE VI.
Effect of Crystalline Streptocin Upon Cell Multiplication of *T. vaginalis.*

Conc. in units/ml in final medium	Population after 48 hr cells/mm³
1.00*	0
0.66	10
0.14	1,010
0	1,600

* At this concentration, the agent is trichomonadicidal.

TABLE VII.
Effect of Sublimation Upon Yield of Streptocin from Crude Extract of *S. griseus* 3533.

	Trichomonadicidal units/mg
Crude ext.	0.96
Crude ext. sublimed	153
Residue from sublimation	0.77

producing strain of *S. griseus*[1,2] streptocin has a greater effect against the avirulent strain of *Mycobacterium* 607 than against the avian strain. It can be crystallized from 1% glacial acetic acid-methanol or from acetone but not from amyl acetate.

A trichomonadicidal unit is defined as that amount of material which when present in 1 ml of STS medium will kill the 75,000 seeded organisms in 48 hours' incubation at 37°C. A comparative study of streptocin and actidione upon different species of trichomonads is presented in Table V. The effect of different concentrations of *streptocin* upon *T. vaginalis* is shown in Table VI.

The active component can be sublimed at 100°C in a vacuum of 1×10^{-5} mm. This sublimation can be accomplished from the initial extract as shown in Table VII. Ac-

TABLE VIII.
Comparative Chemical and Biological Properties of Streptocin and Actidione.

Streptocin	Actidione (3)
Marked antibacterial activities	No antibacterial activity
Limited antifungal activities	Marked activity against fungi
Trichomonadicidal activity 1:29,000 against *T. vaginalis*	Trichomonadicidal activity <1:10,000 against *T. vaginalis*
No effect against *T. gallinae* at 50 μg/ml	Trichomonadicidal against *T. gallinae* at 50 μg/ml
Extremely low toxicity I.V. in mice	MLD_{50} I.V. in mice 150 mg/kg
Insoluble in $CHCl_3$	Soluble in $CHCl_3$
Forms gel with amyl acetate	Recrystallizes from amyl acetate
Stable at pH 11.0	Unstable to alkali
Sublimable	Nonsublimable

TABLE IX.
Antimicrobial Activity of Certain Chromotographic Fractions Other Than Streptocin.

Fraction	Dilution units per 1 mg			
	E. coli	*B. mycoides*	*S. aureus*	*B. subtilis*
10% acetone-benzene to 25% ethanol-acetone	1	24	75	100
Absolute ethanol	3	3	8	24
50% methanol-ethanol	3	3	3	100

tidione could not be sublimed under the same conditions. The crystalline material is soluble in water, methanol, and ethanol but not in chloroform. On standing, the material loses its crystalline structure. It is stable in a pH range of 2.0 to 11.0. It is stable to heat at 100°C for at least 60 minutes. The dry material is stable at 5°C for at least 4 months. Spectrophotometric measurements indicated no absorption maxima between 2200 and 6750 Å.

The comparative chemical and biological properties of streptocin and actidione are shown in Table VIII. These results show that streptocin is distinct from actidione. The antimicrobial spectrum of streptocin is similar to that of crude material isolated from the different strains of *S. griseus,* in that it acts largely upon gram-positive bacteria. However, other chromatographic fractions obtained from the crude material possess similar antimicrobial properties but are not trichomonadicidal. The antibiotic properties of several such fractions are shown in Table IX.

Course of production of streptocin by S. griseus 3533. The course of production of streptocin can be measured by its trichomonadicidal activities. Most of the material containing the trichomonadicidal activity is produced in the pellicles, and stationary cultures give much higher yields than submerged cultures. The results of typical experiments are shown in Fig. 2. Maximum production of the antibiotic is obtained in static cultures on the fifth day, when 76% of the activity is found in the pellicle and 24% in the culture filtrate.

The *Trichomonas foetus* used in these experiments was received from Dr. B. B. Morgan, University of Wisconsin. The authors of the New Brunswick group wish to acknowledge the assistance of Miss Dorothy Smith in testing the activity of the ether extract of pellicle of streptomycin-producing

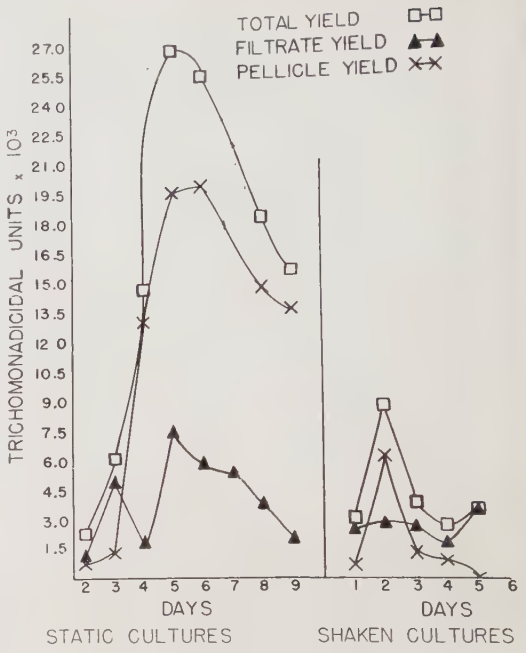


FIG. 2.
Comparison of relative yields of trichomonadicidal activity from the pellicle and filtrate of *S. griseus* No. 3533 grown in static and shaken cultures.

S. griseus against the various strains of *M. tuberculosis.* The authors of the Ortho group wish to acknowledge with appreciation the technical assistance of Mrs. Mary Williams.

Summary. Several antibiotic substances were found in the ether-soluble extract of the mycelium of various cultures of *S. griseus,* which differed in their chemical nature and antimicrobial activities. One of these fractions from *S. griseus* No. 3533, designated as *streptocin,* possesses strong trichomonadicidal properties. It is active against gram-positive bacteria. Small amounts of streptocin are also present in the culture filtrate of the organism.

Streptocin is distinct from actidione in its physical and chemical properties and in the nature of its antimicrobial spectrum.

16910

An Experimental Study of Some Repository Dosage Forms of Penicillin.

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(Introduced by L. E. Arnow.)

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The injection of repository dosage forms of penicillin results in a prolongation of plasma penicillin concentrations. Crystalline sodium or calcium penicillin suspended in peanut oil and beeswax,[1,2] procaine penicillin suspended in oil[3,4] or in water[5] are used for this purpose.

Experimental. During the course of a research program designed to develop an effective suspending vehicle for such repository dosage forms, dogs were given an intramuscular injection of 300,000 units of penicillin in the form of some of these products or of an experimental preparation consisting of procaine penicillin in peanut oil and aluminum monostearate gel, as well as of the non-repository dosage form of sodium penicillin in aqueous solution. Penicillin concentrations of heparinized plasma samples were determined by a modification of the Rammelkamp tube dilution assay method.[6,7] For purposes of arithmetic averaging, amounts of penicillin not detectable by the assay were considered as zero values. Table I lists the average penicillin values found following the injection of each of these products into 5 dogs. It will be seen that, of the preparations tested, the use of the oil-monostearate suspension resulted in the greatest prolongation of detectable levels of plasma penicillin.

It was believed that a test of ability to prolong a period of protection against an experimentally induced infection might offer a more definitive means of evaluation of these preparations than the study of duration of detectable plasma penicillin concentrations. Accordingly, the mouse protection test described below was developed.

Mice were divided into groups corresponding to the number of preparations to be tested. At zero time each mouse received a single intramuscular injection in the left hind leg of 15,000 units (0.05 cc) of the appropriate penicillin suspension. At stated time intervals after this treatment, 10 or 20 mice from each of the original groups were challenged with 1,000 minimum lethal doses of a 6-hour culture of Pneumococcus Type I. Following challenge, the mice were observed for seven days, at the end of which time the final calculations of percentage survival were made.

The effect of penicillin particle size on protective ability was investigated because of the report that oil-beeswax preparations were most effective in prolonging plasma penicillin concentrations when large particle size crystals were used in the suspension.[8] Two preparations of sodium penicillin and an oil-beeswax base were formulated: in one the majority of the crystals measured 50 microns and in the other the majority of the particles measured less than 5 microns. Similarly, large and small particle size samples of procaine penicillin were suspended in oil and aluminum monostearate. Several tests were run on each preparation, and the reported figures represent

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3 Herrell, W. E., Nichols, D. R., and Heilman, Fordyce R., *Proc. Staff Meet. Mayo Clinic*, 1947, **22**, 567.

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5 Whittlesey, P., and Hewitt, W. L., Proc. Soc. Exp. Biol. and Med., 1948, **68**, 658.

6 Rammelkamp, C. H., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 95.

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TABLE I.

Effect of Dosage Form on Average Plasma Penicillin Concentration in Dogs Following a Single Intramuscular Injection (1.0 cc) of 300,000 Units of Penicillin.

Dosage form		Avg plasma penicillin conc. (u/cc) at different hrs following penicillin injection				
Penicillin salt, 300,000 u	Suspending vehicle	1	8	24	72	96
Sodium	Water*	22	0.06	.01		
Procaine	Oil	3.8	1.7	.55	.02	
”	Water	2.7	2.5	.19	.03	
”	Oil-monostearate	2.8	1.0	.51	.12	.09

* Solution.

TABLE II.

Effect of Dosage Form on the % Survival of Mice Receiving a Single Intramuscular Injection (0.05 cc) of Penicillin and Challenged with Pneumococci.

Dosage form			Time (hrs after treatment) of challenge with 1,000 M.L.D. Type I pneumococcus									
			% survival									
Penicillin salt 15,000 u	Suspending vehicle	Particle size	1	4	8	24	48	72	96	120	144	168
Sodium	Water*		100	100	45	0						
Procaine	Oil		100	100	100	13	0					
”	Water		100	100	75	7	0					
Sodium	Oil-beeswax	large				84	47	33	2			
		small				40	17	17	3			
Procaine	Oil-monostearate	large				66	30	3	0			
		small				100	97	87	66	34	13	0

* Solution.

calculations involving at last 20, and in most cases, 40 to 60 mice. Results of these experiments are included in Table II. It will be seen that none of the mice injected with aqueous solutions of penicillin were protected against an infection introduced 24 hours after the prophylactic treatment, but that 13% of the mice receiving procaine penicillin in oil and 7% of those receiving aqueous suspensions of the procaine salt retained sufficient penicillin for a 24-hour period to protect them against the pneumococcus infection given at that time. Large crystal sodium penicillin preparations protected a greater percentage of the test animals than did the small particle suspension, but, in contrast to these results, the small particle procaine penicillin in oil and aluminum monostearate was more effective than was the large crystal preparation, both in increasing the survival percentage and in prolonging the period of protection against infection. These data clearly demonstrate a remarkable superiority of small particle procaine penicillin suspended in oil and aluminum monostearate over all the other preparations tested in this manner.

Discussion. It is believed that the mouse protection test described here is a valid means of comparing the duration of therapeutic action of repository type penicillin preparations, and that it may be used to estimate the probable effectiveness of such preparations in producing prolonged plasma penicillin concentrations in human patients. Such a test has been found to be a useful tool in surveying experimental repository dosage formulas, and may be used as a guide in choosing preparations for clinical investigation.

Summary. A mouse protection test in which penicillin products are used prophylactically against subsequent challenge with Pneumococcus Type I is described. This test was used to evaluate repository type dosage forms of the antibiotic agent for their ability to prolong a period of protection against infection. Of the preparations tested the non-

repository aqueous solution of sodium penicillin was least effective, the suspensions of procaine penicillin in water or in oil were approximately equal in their activity, large particle size sodium penicillin in beeswax and oil was more effective, and small particle procaine penicillin suspended in oil and aluminum monostearate was the most effective preparation tested.

16911 P

Differentiation of Three Groups of Poliomyelitis Virus.*

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In a previous report in which 7 strains of poliomyelitis virus were compared by the method of challenging convalescent monkeys, Kessel and Pait[1] indicated that 4, BK, McK, Ca and Fr constituted one group, which was designated as Group A, or Group I.†

Two of the other 3 viruses studied, the La and Le, were distinct from the above 4 while the seventh, MV, appeared to exhibit an intermediate relationship. In 1946,[2] it was shown that La and Le were different by reciprocal neutralization tests.

Methods. In order to compare these relationships further, 4 of the viruses, McK, MV, La and Le were selected for reciprocal studies by 2 additional methods:

(a) challenge of vaccinated animals.

(b) Neutralization by pooled sera from the vaccinated animals. The vaccination was by the intramuscular route as recommended by Morgan,[3] the following schedule being used, 1.25 cc of 20% virus at 0, 1, 3 and 5 weeks, making a total of 1 g of infected monkey cord received by each animal. All animals were challenged with 100 $P.D._{50}$ of homologous virus by the intracerebral route at the seventh week. All immune animals were then challenged with 100 $P.D._{50}$ of a heterologous virus during the 13th week. Just prior to the heterologous challenge the monkeys were bled and their serums saved for neutralization

TABLE I.
Reciprocal Challenge of Vaccinated Animals. Numerator equals number of monkeys showing symptoms, denominator equals number of monkeys inoculated.

Vaccinated with	Challenged with			
	McK	MV	La	Le
McK	0/12	1/3	4/5	*
MV	4/4	0/12	1/4	5/5
La	5/5	*	0/11	6/6
Le	*	*	2/4	0/4

100 PD_{50} used in challenge.
* Incomplete.

TABLE II.
Reciprocal Neutralization Tests.

Pooled serum from animals vaccinated with	Virus used in neutralization test			
	McK	MV	La	Le
McK	0/4	3/5	5/5	5/5
MV	4/4	0/4	0/4	4/4
La	5/5	2/4	0/5	4/5
Le	4/4	5/5	4/5	0/5
Controls	6/6	5/6	5/6	6/6

100 PD_{50} of virus was used.

* Aided by a grant from the National Foundation for Infantile Paralysis.

1 Kessel, J. F., and Pait, Charles E., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 606.

† More recently 5 additional strains, Mi, from Dr. Thomas Francis, Jr.; Gu, isolated in Los Angeles; Ko, and Cp, from Dr. William M. D. Hammon; and Br, from Dr. David Bodian, have been tested and found to belong to Group I.

2 Kessel, J. F., Moore, F. J., and Pait, Charles E., *Am. J. Hyg.*, 1946, **43**, 82.

3 Morgan, I. M., Howe, H. B., and Bodian, D., *Am. J. Hyg.*, 1947, **45**, 379.

tests, a separate serum pool being made from monkeys vaccinated with each strain. In the neutralization test, the final virus concentration was 100 $P.D._{50}$ per cc and serum dilution 1:2. This mixture was incubated for 4 hours in the refrigerator just prior to intracerebral inoculation of 5 monkeys with 1.0 ml each.

Results. Tables I and II summarize the results.

Conclusions. These results in conjunction with those of the previous study indicate that 3 groups of poliomyelitis virus have been demonstrated. I. One group encompasses at least 9 viruses compared in this study: Bk, McK, Ca., Fr, Mi, Gu, Ko, Cp. and Br. This group, which includes the greatest number tested to date has been designated tentatively as Group I. II. Another group so far comprises the La and MV viruses. III. A third virus, Le, differs sufficiently from the above two groups to represent a third group.

16912 P

Relationship of Body Specific Gravity to Body Fat and Water Content.*†

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It has been demonstrated by Behnke and his coworkers[1-3] that the specific gravity of the human body can be used as an index of the proportion of fat present. Rathbun and Pace[4,5] measured the specific gravity of eviscerated guinea pigs and also analyzed the bodies chemically for the fat content. They found that the body specific gravity of the animals decreased as the fat content increased, and that the range of specific gravity for the guinea pigs closely approximated that found by Behnke in his series of normal human males. The relationship between body specific gravity of the eviscerated guinea pig and the proportion of body fat was found to be a very exact one and agreed closely with the theoretical equation:

$$\% \text{ fat} = 100 \left(\frac{5.548}{\text{specific gravity}} - 5.044 \right)$$

The use of body specific gravity as an index of the amount of body fat in humans has been examined further in this laboratory. In an effort to describe more accurately the relationship which exists between metabolic rate and age, studies are being carried out to ascertain whether the "lean body mass," *i.e.*, body weight minus the fat content, may not be a better standard of reference for the metabolic functions of the body than surface area or weight of the body.

The difficulties encountered in weighing elderly people under water, a part of the procedure for measuring specific gravity of the body, were so great that it was decided to try to measure body water instead and calculate the lean body mass from this determination. A chemical method for the measurement of total body water, by the use of antipyrine (1-phenyl-2:3-dimethyl-5-pyrazolone) was then developed in this laboratory and the values obtained agreed closely with simultaneous measurements of body water by deuterium oxide.[6]

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

† Part of the expenses of this study were defrayed by the Josiah R. Macy, Jr. Foundation.

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2 Behnke, A. R., Feen, B. G., and Welham, W. C., *J.A.M.A.*, 1942, **118**, 495.

3 Welham, W. C., and Behnke, A. R., *J.A.M.A.*, 1942, **118**, 498.

4 Rathbun, E. N., and Pace, Nello, *J. Biol. Chem.*, 1945, **158**, 667.

5 Morales, M. F., Rathbun, E. N., Smith, R. E., and Pace, Nello, *J. Biol. Chem.*, 1945, **158**, 677.

TABLE I.
Relationship of Body Specific Gravity, Body Fat, and Body Water.

Subject	Specific gravity	% body fat		% body water		% water
		from spec. grav.	from antipyrine	from spec. grav.	from antipyrine	from lean body mass
1	1.021	39.0	41.5	44.4	43.4	71
2	1.032	33.2	29.0	49.3	51.8	77
3	1.032	27.5	30.4	53.0	50.7	70
4	1.044	27.0	25.2	53.4	53.2	73
5	1.045	26.5	31.0	54.0	50.0	68
6	1.057	20.5	17.2	58.2	60.5	76
7	1.061	18.5	16.3	58.6	58.8	72
8	1.061	18.5	20.4	58.6	58.0	72
9	1.064	17.0	19.9	59.6	58.5	72

Thus in the same individuals it became possible to measure body water directly by a chemical method and by specific gravity, using Pace and Rathbun's formula.[7] This circumstance permitted a verification of both methods in a number of individuals. Pace *et al.* have previously shown a good check in one individual between the calculation of body water made from specific gravity and direct measurement by tritium oxide.[8]

Methods. The specific gravity of nine normal individuals was determined by weighing them in air and under water according to Behnke's procedure. On the same day, total body water of the subjects was measured by the use of antipyrine. In one individual, total body water was simultaneously determined by the use of both antipyrine and deuterium oxide, with good agreement by the two methods. Body fat and body water were then calculated or measured by two independent procedures.

Body fat was calculated from specific gravity[4,5] and from the antipyrine figure for total body water

$$\% \text{ fat} = 100 \left(\text{body wt} - \frac{\text{wt body water}}{0.73} \right)$$

on the assumption that the defatted tissues of the body contain on the average, 73% water.

Body water values were ascertained by direct measurement with antipyrine and calculated from specific gravity

$$\% \text{ body water} = 100 \left(4.424 - \frac{4.061}{\text{sp.gr.}} \right)$$ [7]

Results. The figures for per cent body fat calculated from specific gravity and from total body water determined by antipyrine agree fairly well (Table I) for the 9 individuals studied.

Likewise, there is good agreement of the values for per cent body water calculated from specific gravity and estimated chemically by the use of antipyrine (Table I).

In these tables the subjects are listed in order of increasing specific gravity. The inverse relationship of body water to body fat is readily noted; as the body specific gravity increases, total body water increases, while body fat decreases. Furthermore, the water content of the lean body mass is fairly constant.

Discussion. The fact that body specific gravity can be used to predict fat and water content in normal humans has received scant notice in the recent literature. The concept presented by Behnke and his co-workers that fat tissue is the chief variable of the body and that the fat-free tissue of the body or "lean body mass" is relatively constant in composition, is an important one. Variability in total body water depends, from this point of view, largely on the amount of body fat. This relationship seems not to have been widely appreciated.

In the literature, figures for the proportion of body water have run from 59.3% reported

6 Soberman, R., Brodie, B. B., Levy, B., Axelrod, J., Hollander, V., and Steele, J. M., *J. Biol. Chem.*, in press.

7 Pace, Nello, and Rathbun, E. N., *J. Biol. Chem.*, 1945, **158**, 685.

8 Pace, Nello, Kline, L., Schachman, H. D., and Harfenist, M., *J. Biol. Chem.*, 1947, **168**, 459.

as the result of a desiccation experiment[9] to 72.3 as measured by use of deuterium oxide.[10] The variations may, of course, have been due to the employment of different methods, but in the light of the present study, seem more likely to be the result of varying degrees of obesity.

These data serve to emphasize the necessity for taking into account the amount of adipose tissue of the body before comparison of the proportion of body water between individuals has much significance. They serve also to corroborate the usefulness of specific gravity as a measurement of the proportion of fat and water in the body.

Summary. 1. Body fat and water can be calculated from body specific gravity with considerable accuracy.

2. Use of an independent method of measuring body water clearly shows the close inverse relationship of per cent body fat and per cent body water.

3. The proportion of water in the body is highly variable unless it is expressed in terms of fat-free tissue (lean body mass.)

[9] Gregersen, M. L., MacLeod's Physiology in Modern Medicine, 8th ed., 1938, p. 903.

[10] Moore, F. D., *Science*, 1946, **104**, 157.

16913

Assay of Aureomycin in Body Fluids: Observations on Individuals Receiving Aureomycin.*

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In order to place therapy with the promising new antibiotic, aureomycin, on a rational basis, the development of a satisfactory method of assay in body fluids is necessary. It is the purpose of the present communication to describe such a method of assay and to report some of the initial results from the application of this method in the study of the use of aureomycin.‡

At the time of the preparation of this report, only two groups of investigators have reported the results of attempts to assay the drug. The first group, using a *B. subtilis*-like organism, reported levels in humans following ingestion of various amounts of the drug.[1] The results were expressed as "units" which represented one-seventh of a microgram of the antibiotic. Paine, Collins, Finland, and Wells,[2-4] in studying aureomycin, reported on plasma levels of the drug as well as a quantitative method applicable to the urine. They stated that their method for determining the plasma levels was not satisfactory.[3,4] The urine levels were expressed as dilutions of urine which inhibited the test organism, streptococcus No. 98, rather than in terms of concentration of the drug.

Method. In this laboratory a modification of the Rammelkamp tube dilution technic[5]

* Aided by grants from C. S. Howard Donation and the Fleischner Endowment Fund.

† This laboratory is supported by the Divisions of Medicine and Pediatrics of the University of California Medical School, and by the Department of Public Health of the City and County of San Francisco.

‡ The aureomycin used in this investigation was provided by Lederle Laboratories, Division of the American Cyanamid Company.

[1] Cox, H. R., personal communication.

[2] Paine, T. F., Collins, H. S., Finland, M., *J. Bact.*, 1948, **56**, 489.

[3] Collins, H. S., Wells, E. B., Paine, T. F., Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 174.

[4] Finland, M., Collins, H. S., and Paine, T. F., *J.A.M.A.*, 1948, **138**, 946.

[5] Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

TABLE I.
Composition of Study Group Presented According to Route, Dose, and Period When Assay Was Done.

Route	Dose	Assays following initial dose		Assays following subsequent doses	
		No. persons	No. determinations	No. persons	No. determinations
Oral	0.16-0.6 g	1	1	5	10
	1.0 "	11	36	13	35
	1.5-2.5 "	2	8	2	7
I.V.	50 mg	9	47	2	4
	100 "	1	3	1	5
I.M.	50 "	5	12	—	—
	100 "	1	4	2	4
	200 "	1	1	—	—

which has been used for several years for the assay of penicillin and streptomycin in body fluids, has been applied to the assay of aureomycin. The method has proved practical and forms the basis for the present report.

Technic of the Test; Test Organism. The bacterium used in the test was a strain of beta hemolytic streptococcus (JB) which has been used in this laboratory for antibiotic assay for the past 5 years. This strain is sensitive to 0.0039 unit of penicillin per ml and to 0.015 μg of streptomycin per ml. A 24-hour culture of this organism in brain-heart infusion broth was centrifuged in a Hopkins tube[6] and then diluted with a buffer broth to be described below to make a final concentration of 4000 organisms per 0.5 ml of the broth.

Media. The broth used for the assay was a beef heart infusion (Difco) broth buffered with sodium phosphate to a pH of 7.6 and with 10% neopeptone. Ascitic fluid was added at the time of the test to a final concentration of 20%.

Serial Dilution of the Unknown Fluid. The serum or other body fluid was stored at about –40°C unless tested immediately. Fluids, such as urine, which were not sterile were filtered through Seitz pads. A series of 8 to 10 tubes were set up, each containing 0.2 ml of the buffer broth. To the first of these was added 0.2 ml of the material to be tested and serial 2-fold dilutions were made. The broth suspension of the test organism, in a volume of 0.5 ml, was then added to each tube, making a final volume of 0.7 ml. The tubes were well shaken and placed in an incubator at 37°C for 18 hours. The end-point of the test was taken as the highest dilution of the unknown material showing no growth visible to the unaided eye.

Control. With each assay, the test organism was set up with a known concentration of aureomycin which was prepared by diluting a standard solution of the drug. This standard solution contained a concentration of 10 μg by weight per ml and was stored between tests at –40°C. In the control set-up, 0.2 ml of this solution, containing 2 μg, was diluted in 2-fold steps with the buffer broth through 8 tubes. To each of these was added 0.5 ml of the test organism suspension. The final concentration of aureomycin in these tubes was therefore from 1.0 μg per ml down to 0.007 μg per ml. In this laboratory the standard organism used usually was inhibited in the tube containing 0.031 μg per ml and occasionally varied one tube in either direction, or from 0.062 μg per ml to 0.015 μg per ml.

As a further control over the technic, a known concentration of aureomycin, 5 μg per ml, was included in each run and was diluted as an unknown. In any run where this gravimetric value did not check with the bioassayed value, the results of the determinations on the unknown were considered inaccurate and the materials retested.

Example of Calculation. If, in determining the level of aureomycin in serum, the growth was inhibited up to a dilution of 1:8 and the control series showed the test organism to be inhibited at a concentration of 0.031

[6] Todd, J. C., Sanford, A. H., Clinical Diagnosis by Laboratory Methods, W. B. Saunders, 1935, p. 703.

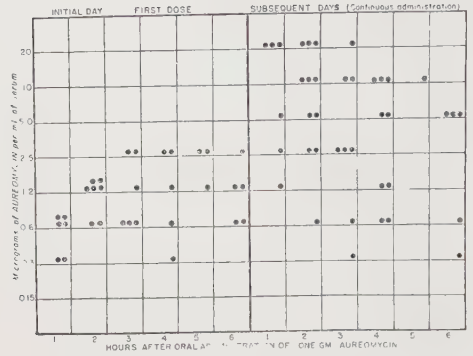

FIG. 1.

Graph illustrating blood aureomycin concentrations following an initial oral dose of one gram, as compared with blood levels following subsequent oral doses of one gram every 4 to 6 hours in patients receiving continuous therapy.

μg per ml, then the serum level was $8 \times 0.031 \times 5$ or 1.24 μg per ml of serum.

Study Group. Thirty individuals receiving aureomycin in a variety of doses and by several routes were studied in the present investigation, as presented in Table I. These individuals included patients under treatment for various infectious diseases and a group of normal individuals.

Results: Serum Levels Following Administration by Various Routes. Over the period of 6 months during which this method of assay has been used in this laboratory, certain general observations have been made. First, the test organism has not shown significant variation in its aureomycin sensitivity as determined by the control assay with known concentrations of the antibiotic. Retesting of serum specimens which had been stored over a period of 4 months at –40°C, showed no deterioration of the antibiotic content. No deterioration of the antibiotic in serum, plasma, or whole blood stored at 4°C for as long as 50 hours has been observed, although this possibility remains.[2] Therefore, all serum specimens were frozen at –40°C as soon as possible after being obtained.

Oral Route. Following the oral administration of an initial dose of 1.0 g of aureomycin in adults, significant concentrations of the drug were present in the blood within one hour, and the maximum concentrations were reached within 2 to 4 hours. The results of serial determinations on a group of 11 such individuals is presented in Fig. 1. The peak concentrations found in different individuals fell between 0.6 and 2.5 μg per ml of serum. Measurable amounts of the drug persisted in the serum for at least 6 hours, and in many individuals no decrease in concentration was observed at that time.

In most individuals to whom aureomycin was administered on a continuous schedule every 4 or 6 hours, the levels of the drug tended to increase gradually (Fig. 1). This was noted as early as after the second dose of the drug, but usually was more marked after several days. In some cases the later levels exceeded the earlier ones by many fold.

When the drug was given in doses of 0.5 or 1.5 g, the blood concentration did not appear to be affected to a degree proportional to the differences between these doses and the 1.0 g dose, although some difference was usually observed. Doses of aureomycin in children reduced roughly according to body weight appeared to produce concentrations comparable to those attained in adults following administration of 1.0 g.

Intravenous Route. Within 5 minutes following the intravenous administration of 50 mg of aureomycin dissolved in 5.0 ml of 0.784% Na_2CO_3 solution, drug concentrations were reached which were equal to or exceeded the maximum values obtained following oral administration of 1.0 g. Results of serial determinations of drug concentrations in 11 individuals following this dose are presented in Fig. 2. Serum levels rapidly declined dur-

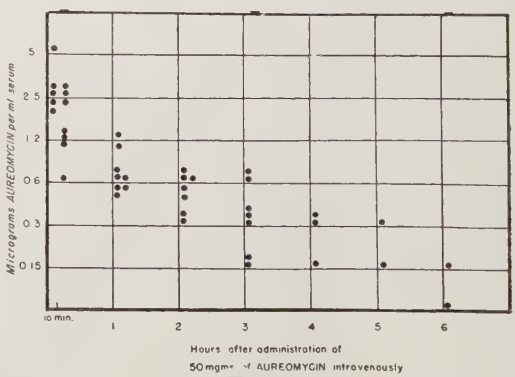

FIG. 2.

Graph illustrating blood aureomycin concentrations following the initial intravenous administration of 50 mg.

ing the first hour and decreased slowly thereafter. Measurable concentrations were present as long as 6 hours after administration.

Intramuscular Route. When the drug was administered by the intramuscular route in doses of 50 to 200 mg dissolved in 5.0 ml of a Sorensen phosphate buffer (pH 7.2) and mixed with an equal volume of 2% procaine, measurable levels were rarely noted during the following 4 hours. In only one of 21 determinations was a concentration greater than 0.15 μg per ml observed.

Urine Levels. Aureomycin concentrations were readily measured in the urine using this method of assay. Following doses of the order described above, concentrations of from 5 to 50 μg per ml were observed. Much smaller amounts were observed during the 4-hour period following the ingestion of 1.0 g than following the intravenous administration of 50 mg.

Cerebrospinal Fluid Levels. From 1 to 3 hours following the ingestion of 1 g, no measurable amount of aureomycin was observed in the cerebrospinal fluid in 3 assays on 2 individuals with meningeal irritation. In one normal child aureomycin was not demonstrated in the cerebrospinal fluid after the ingestion of 2 grams over a period of 24 hours.

Sensitivity of Organisms. The sensitivity of a variety of pathogens isolated in this laboratory varied from 0.017 μg per ml to 285 μg per ml. Most organisms tested were sensitive to less than 1.0 μg per ml.

Discussion. The tube dilution method of antibiotic assay in body fluids, although having certain disadvantages, represents a practical and convenient procedure. Variations in the end-point may occur with variation in the size of the inoculum of test organism.[2] These, however, are minimized by the use of a constant, small inoculum. Although the use of the inhibition of visible growth at 18 hours of incubation as the end-point may not coincide with complete inhibition as evidenced by subculture, neverthless, this discrepancy is relatively constant.

One of the problems inherent in any method of assay of an antibiotic is the rate of deterioration during handling and under conditions of incubation. This factor may be more important in the case of aureomycin than in the assay of penicillin and streptomycin.

Since high blood concentrations were often not reached for several days when the drug was given by mouth, a combination of the more efficient intravenous route and the more convenient oral route may be advisable in the treatment of many severe acute infections. The intramuscular route in our hands has been of very limited value because measurable serum levels were so rarely obtained. Frequent painful local reactions have occurred.

Summary. 1. A serial tube-dilution method of determinations of aureomycin concentrations in body fluids using an hemolytic streptococcus as the test organism is described.

2. Serum levels of aureomycin ranged from 0.3 μg to 2.5 μg during a 6-hour period following the initial oral dose of 1 g.

3. Serum levels or aureomycin following doses of 1 g on subsequent days in patients receiving continuous therapy ranged from 0.3 μg to 20 μg during a 6-hour period.

4. Serum concentrations of aureomycin following the intravenous administration of 50 mg ranged from 0.6 to 5 μg 5 minutes after injection and declined gradually over a 6-hour period.

16914

Hypothermia in the Mouse as a Bio-Assay of Endotoxin Protection Factor in Impure Penicillin.*

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From the Department of Medicine, University of Chicago.

In seeking an improved method of bio-assay of the endotoxin protection factor of impure penicillin,[1-4] it was found, as reported by Beck[5] and Zahl and Hutner,[6] that mice given bacterial endotoxins were unable to maintain their body temperature. In addition, we found that this hypothermia was less marked in animals treated with impure penicillin and that the degree of hypothermia was a function of the amount of impure penicillin administered.

Methods. Young adult (20 g) mice were kept in a constant temperature room at 24°C for at least 2 days before and during the entire time of each experiment. At the time the endotoxin was given the mice were placed in individual compartments which prevented them from huddling together and permitted the course of an individual mouse to be followed throughout the experiment.

0.5 ml of impure penicillin, containing 10,000 units of crystalline penicillin per ml, was given intraperitoneally at 20, 18 and 2 hours before the injection of the endotoxin.

The endotoxin, prepared from *Salmonella acrtrycke* as previously described[7] was administered intraperitoneally as an endotoxin-saline mixture, the total volume of fluid being 1.0 ml in all cases.

TABLE I.
Method of Median Calculation.

Hr after endotoxin	Protected mice 22 hr	26 hr	28 hr	30 hr
	37.1	37.6	38.0	38.2
	36.9	37.5	37.7	37.7
	36.7	37.4	37.5	37.7
	36.3	37.0	37.4	37.3
	35.9	36.8	37.2	37.2
	35.6	36.6	36.8	36.7
	35.3*	35.8*	36.0*	36.6*
	34.4*	35.7*	35.8*	36.3*
	34.3*	35.2*	35.7*	36.0*
	33.3*	35.0*	35.6*	35.8*
	30.9*	32.3*	34.1*	34.7*
	30.7*	31.3*	32.8*	32.1*
Median temp.	35.4	36.2	36.4	36.7
	Unprotected mice			
	32.8	33.3	34.6	35.0
	32.3	32.7	33.2	33.4
	29.4	31.3	30.6	26.6
	27.9	27.4	27.5	26.5
	27.9	27.1	26.9	26.4
	27.4	26.5	26.5	26.1
	26.9*	26.4*	26.4*	25.9*
	26.5*	26.3*	26.3*	25.9*
	26.0*	25.3*	26.1*	25.5*
	25.8*	25.2*	25.2*	Dead*
	25.7*	Dead*	Dead*	Dead*
	25.6*	Dead*	Dead*	Dead*
Median temp.	27.1	26.5	26.5	26.0

* Mice with body temperature below the median of the group.

* This investigation was supported (in part) by a research grant from the Antibiotics Study Section of the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service, and (in part) by the A. B. Kuppenheimer Foundation and the Douglas Smith Foundation of the University of Chicago.

† Research Fellow, U. S. Public Health Service.

‡ Douglas Smith Foundation Fellow in Medicine.

The impure penicillin was supplied through the courtesy of Dr. Robert D. Coghill of the Abbott Laboratories. The crystalline penicillin was supplied by Abbott Laboratories, Commercial Solvents Corporation, Lederle Laboratories, and Schenley Laboratories, Inc.

1 Boor, A. K., and Miller, C. P., *Science,* 1945, **102**, 427.

2 Miller, C. P., and Boor, A. K., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 18.

3 Boor, A. K., Miller, C. P., and Hawk, W. D., *Fed. Proc.,* 1947, **6**, 240.

4 Miller, C. P., Hawk, W. D., and Boor, A. K., *Science,* 1948, **107**, 118.

5 Beck, L. V., A.A.A. S. Symposium on Approaches to Tumor Chemotherapy, 1947, p. 265.

6 Zahl, P. A., and Hutner, S. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 156.

7 Boor, A. K., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 168.

8 Nielsen, E. T., *Acta Medica Scand. Supplementum,* 1938, **90**, 168.

The rectal temperatures were determined by the use of a copper-constantan thermocouple and a galvanometer. One junction was inserted a distance of 3 cm into the mouse's rectum and the precautions advised by Nielsen[8] regarding its position were observed. The other junction was placed in a constant temperature water bath.

As a routine schedule, 4 temperature determinations were made at approximately 21, 24, 27 and 30 hours after the endotoxin was injected. Less than 4 determinations increased the standard error. Twelve animals in each group were found to be sufficient for a fairly quantitative result, giving a standard error of about 0.8°C for the average of 4 determinations. This meant that a difference of approximately 3°C between the two groups of mice was significant.

The median temperature of each group of mice was chosen for the final calculation rather than the mean temperature because the loss of a mouse by death raised the mean temperature

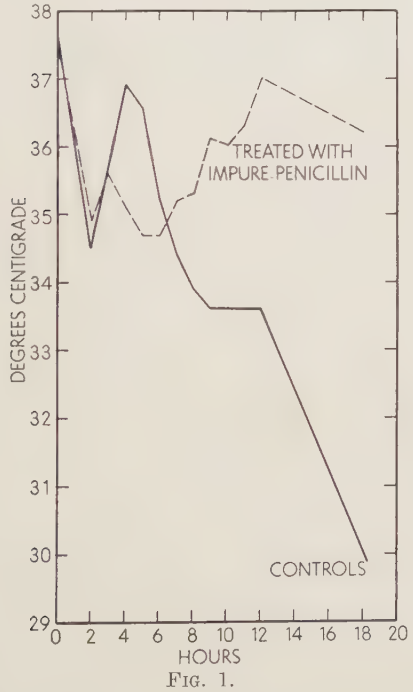

FIG. 1.

Median temperatures of mice injected with endotoxin.

Note the two phases of hypothermia after the injection of the endotoxin; the impure penicillin protects against the later more important phase of hypothermia.

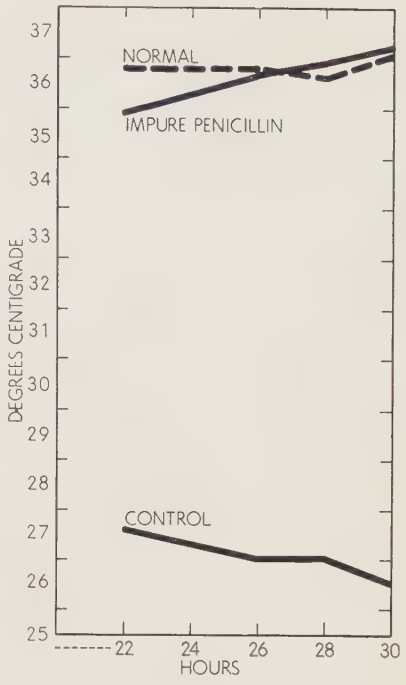

FIG. 2.

Median temperatures of mice injected with endotoxin.

Comparison of the median temperature of treated (impure penicillin) and control mice injected with endotoxin; and normals receiving no injection.

of the group. The dead mice were, therefore, listed at the bottom of their respective groups when the medians were determined and a more satisfactory computation resulted. This was felt to be a justifiable procedure since among 457 mice which died (of a total of about 1,500) 88% were animals with temperatures previously below and only 12% at or above the median. A sample experiment and calculation is given in Table I.

Experimental. Fig. 1 presents the results of a typical experiment. Two groups of mice were used: one protected by impure penicillin, the other untreated. Both groups were given 0.05 ml of the endotoxin intraperitoneally.

The results of an experiment using the routine procedure described under "methods" are shown in Fig. 2; the data appear in Table I. The temperatures of normal untreated mice kept under the same conditions and determined at the same time are also shown in this figure.

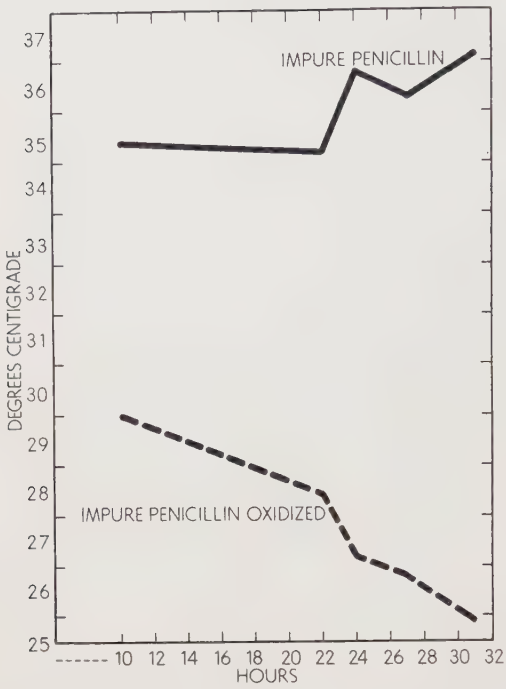

FIG. 3.
Median temperatures of mice injected with endotoxin.

Differences between protected and unprotected groups of mice as shown in Fig. 1 and 2 have been found in 25 separate experiments, using 1,140 mice in which temperatures were taken at least 4 times.

We have found a rough correlation between the degree of protection of a given substance computed by the LD_{50} and the anti-hypothermic effect. In order to investigate the correlation between the two types of experiments, the protective activity was destroyed by oxidation with hydrogen peroxide. The LD_{50} of the endotoxin in mice treated with oxidized impurity was 0.065 ml; in mice protected with unoxidized material the LD_{50} was 0.35 ml. The results of the two subsances in a temperature experiment are shown in Fig. 3.

The impure penicillin did not protect against the hypothermic effects of alpha naphthyl-thio-urea or crystalline insulin.

In order to obtain some quantitative results, an experiment consisting of 18 groups of 12 animals each was performed. The results can be seen in Fig. 4. This graph shows that the method can be used for a quantitative assay of the protective material; however, only a narrow range of the concentration of the protective material can be measured with one dose of endotoxin. In actual practice it was more economical of materials to use a constant dose of endotoxin and various dilutions of the protective factor. The curve shown in Fig. 5 plots the averages of 4 median temperatures of 4 groups of mice treated with varying concentrations of impure penicillin and injected with a standard dose of endotoxin.

Discussion. The greatest error in the method is the biological variation in the mice which necessitates the use of 12 animals in each group. The method also requires that the mice be kept in a constant temperature room and preferably in compartment cages to keep them separated from one another. It has the advantage of greater economy of mice and materials than a satisfactory determination of LD_{50} and is much more quantitative than the latter.

Autopsies, including microscopic examination, failed to disclose any significant trauma

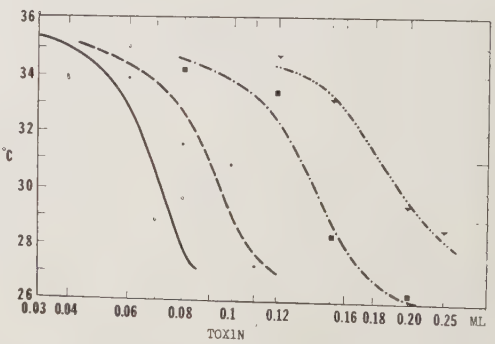

FIG. 4.
Median temperatures of mice injected with endotoxin.

This figure shows the effect of varying doses of a standard preparation of impure penicillin. Each point on the curves represents the average of 4 median temperature determinations between 20 and 30 hours after the administration of endotoxin.

—— Penicillin and saline treated controls.
– – – Treated with one part impure penicillin and 3 parts water.
—•—•— Impure penicillin diluted with an equal volume of water.
••—••— Impure penicillin undiluted.

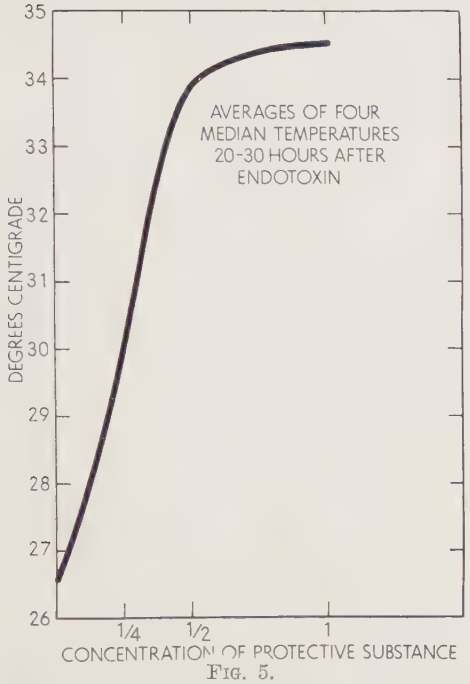

FIG. 5.

Effect of varying doses of impure penicillin. One can distinguish between the protective effect of penicillin and saline, impure penicillin diluted to ¼, and impure penicillin diluted to ½ by using 0.1 ml of endotoxin, as was done in this experiment. In order to distinguish half strength from undiluted material, one must dilute further.

from the insertion of the junction into the mouse.

Hypothermia may be regarded as the physiological response of the mouse to the endotoxin, comparable to fever in most other animals. That it is not merely an ante-mortem phenomenon is evidenced by the fact that less than one-third of the mice die in an experiment. In some instances mice have recovered after their temperatures have been as low as 26°C for 8 to 12 hours.

Summary and Conclusions. The endotoxin of *Salmonella aertrycke* injected intraperitoneally into mice caused a marked fall in body temperature. A certain phase of this hypothermia could be prevented to a significant degree by previous administration of impure penicillin. This phenomenon has been utilized for the quantitative assay of impure penicillin.

A correlation was demonstrated between the antilethal and the anti-hypothermic action of the impure penicillin against bacterial endotoxin in mice.

We are grateful to Dr. John F. Perkins of the Department of Physiology, University of Chicago, for the use of the thermocouple and galvanometer.

16915

Effect of Nicotinic Acid Monoethylamide on the Liver of the Young Rat.

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It has been reported by Brazda and Coulson[1] that a diet containing 1% coramine (nikethamide) produces a rapid and significant increase in the liver weight-body weight ratio of young rats. Nicotinamide, under similar conditions, produces no significant increase.[2] Inasmuch as coramine is disubstituted on the amide nitrogen whereas nicotinamide is unsubstituted it was decided to determine the effect produced by nicotinic acid monoethylamide in which the amide nitrogen is only monosubstituted. This communication reports the results obtained by use of this compound in the diet.

Experimental. The procedure employed was the same as that used in the study of the effect of coramine.[1] The nicotinic acid monoethylamide was mixed into the basic diet

1 Brazda, Fred G., and Coulson, R. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 37.

2 Coulson, R. A., and Brazda, Fred G., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 1.

TABLE I.*
Effect of the Ingestion of Nicotinic Acid Monoethylamide on the Liver of Young Rats.

No. days	Rat final wt	Liver wt wet	Liver wt dry	Liver wt as % body wt	Liver fat % wet wt	Liver fat % dry wt	Liver % solids	Gain in wt g/day
				Control				
5	54.7	2.72	0.80	4.98 ± 0.09†	3.87	13.27	29.21	+3.21
9	70.0	3.36	1.06	4.78 ± 0.09	4.22	13.38	31.47	+3.30
28	148.9	7.74	2.29	5.18 ± 0.13	3.22	10.89	29.60	+3.79
				1% nicotinic acid monoethylamide				
5	36.9	2.05	0.61	5.34 ± 0.12	4.28	14.28	29.53	+0.34
9	55.9	3.00	0.93	5.38 ± 0.10	5.14	16.58	30.91	+1.54
28	108.8	5.75	1.87	5.30 ± 0.11	4.50	13.65	32.78	+2.36

* Each figure in the table represents the average value obtained from 5 males and 5 females.

† P.E. $= \pm 0.6745 \sqrt{\frac{\Sigma(v)^2}{n(n-1)}}$

at a level of 1%. Each of the 3 experimental groups consisted of 10 weanling rats, 5 males and 5 females, each from a different litter. One group was sacrificed for analysis at 5 days, a second at 9 days and the third at 28 days. The analytical procedures were identical with those reported previously.[1] The results appear in Table I.

Microscopic examination of hematoxylin-eosin preparations of sections of livers taken from the experimental animals yielded no unusual pathological findings. Binucleate cells and mitotic figures were seen in slightly greater numbers than in the control series. The difference was not great enough to be of true significance.

Discussion. The ingestion of nicotinic acid monoethylamide at a dietary level of 1% by the young rat failed to produce the significant increase in the liver weight-body weight ratio evoked by coramine.[1] It has been shown by Ellinger and Coulson[3] that coramine is converted quite slowly into nicotinamide in the human. Whether this involves de-ethylation or hydrolysis to nicotinic acid and subsequent conversion to nicotinamide is not known. It would appear from the results that the nicotinic acid monoethylamide is possibly more readily converted to nicotinamide than is coramine. That it is thus converted in the human has been demonstrated by Ellinger and Coulson.[3] However, since the main purpose of the experiment was to determine the effect of the compound on the liver no evaluations of the rate of conversion to nicotinamide were carried out.

Conclusion. Although the mechanism by which coramine produces its effect on liver size remains unknown it is quite apparent that disubstitution of the amide nitrogen is essential. Monosubstitution of the amide nitrogen with an ethyl group either cannot produce a significant effect or else this compound is so readily converted to nicotinamide, which has no such action, that an effective concentration of the necessary substance cannot be achieved.

[3] Ellinger, P., and Coulson, R. A., *Biochem. J.*, 1944, **38**, 265.

16916

The Effect of Feeding Excess Glycine, L-Arginine, and DL-Methionine to Rats on a Casein Diet.*

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Previous reports[1-5] have demonstrated, that in rats and dogs, the addition of different levels of DL-methionine to a casein diet, affects the nitrogen balance index and may alter the physiological state of the animal. Low levels of methionine elevate the nitrogen balance index of casein, causing increased retention of nitrogen; intermediate levels do not alter the nitrogen balance index but cause weight loss and kidney hypertrophy; high levels depress the nitrogen balance index, there is a tearing down of body tissue, severe weight loss, kidney hypertrophy and an increase in creatine and creatinine excretion. It has also been shown[5] that excess L-arginine added to the high level DL-methionine diet, is effective in preventing, in large measure, kidney hypertrophy and also counteracts the lowering of the nitrogen balance index and weight loss.

The work described in this paper was designed to explore the effect of adding excess glycine alone and in combination with methionine and arginine, upon the nitrogen balance index of casein, organ and body weights, and creatine and creatinine excretion.

Methods. The methods for determining nitrogen balance indexes were the same as those described for the dog by Allison, Anderson, and Seeley[6] and for the rat by Brown.[4] These indexes (K) are functions of the rate at which the protein stores of the body are being filled by a given protein source and are calculated from the following equation: $NB = K\ (AN) - NE_0$ where NB is nitrogen balance, AN is absorbed nitrogen and NE_0 is the excretion of nitrogen on a protein-free diet. Mature, male rats of Sherman strain weighing approximately 250 g were used in the experiments. The rats were divided into 6 groups, 10 in a group and fed the following nitrogen sources. Values are on a dry weight basis.

Group	Casein
I	12% (controls)
II	12% + 4.8% DL-methionine
III	12% + 4.8% glycine
IV	12% + 4.8% glycine + 4.8% DL-methionine
V	12% + 4.8% glycine + 4.8% DL-methionine + 1.7% L-arginine
VI	12% + 4.8% glycine + 1.7% L-arginine.

The diets were, in addition, adequate with respect to carbohydrates, fats, minerals and vitamins. All the rats were pair fed with those fed excess methionine (Group II) since this group restricts their food intake. The experiment was continued for 20 days; the urine and feces were discarded for the first 4 days and then collected, thereafter, in four, 4-day periods. At the end of 20 days the animals were autopsied and the livers and kidneys dried at 95°C and analyzed for nitrogen. All deviations listed are standard errors.

Results and Discussion. In Table I are recorded the nitrogen intakes, the urinary and fecal nitrogen excretions, the nitrogen balances and the nitrogen balance indexes for the 6 groups.

The values for nitrogen balance index in

* This work was supported by a grant from the Office of Naval Research and Development. Some of these data were included in a paper read before the Biological Division of the American Chemical Society, Washington, D.C., 1948.

1 Miller, L. L., *J. Biol. Chem.*, 1944, **152**, 203.

2 Allison, J. B., Anderson, J. A., and Seeley, R. D., *J. Nutrition*, 1947, **33**, 361.

3 Brush, H., Willman, W., and Swanson, P., *J. Nutrition*, 1947, **33**, 389.

4 Brown, J. H., Thesis, Rutgers University Library (1948), in preparation for publication.

5 Brown, J. H., and Allison, J. B., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 196.

6 Allison, J. B., Anderson, J. A., and Seeley, R. D., *Ann. N. Y. Acad. Sci.*, 1946, **47**, 241.

TABLE I.
Nitrogen Intake and Excretion of Rats Fed Various Nitrogen Sources.
Averages of 20 values obtained on 10 rats in each group.

Group	Ingested N mg/kg/day	Urinary N mg/kg/day	Fecal N mg/kg/day	N balance g N/kg/day	N balance index
I	397.4	250.2	108.1	+.039	.87
II	537.8	422.8	82.0	+.034	.59
III	600.8	434.0	114.6	+.052	.61
IV	721.5	596.1	96.0	+.029	.55
V	836.0	623.0	95.0	+.118	.49
VI	745.0	469.6	117.2	+.159	.64

TABLE II.
Weight Changes and Creatinine and Creatine Excretions of Rats Fed Various Nitrogen Sources.
Average on 10 rats.

Group	Wt change (avg 20 days), g	Creatinine mg/kg/day	Creatine, mg/kg/day
I	7.4 ± 1.9*	25.8 ± 1.1	5.7 ± 1.0
II	—36.6 ± 6.1	28.9 ± 2.1	9.4 ± 1.6
III	+ 1.2 ± 3.6	26.7 ± 1.3	7.6 ± 0.5
IV	— 1.0 ± 3.6	30.5 ± 1.3	8.0 ± 0.9
V	— 5.0 ± 5.1	30.2 ± 1.2	12.5 ± 1.4
VI	— 5.1 ± 2.1	28.4 ± 1.1	10.3 ± 2.1

* Standard error.

Table I are calculated, relative to that of the casein fed controls (Group I). Examination of Table I shows that the addition of extra nitrogen in methionine (Group II), or glycine (Group III), or methionine plus glycine (Group IV) causes no significant change in the nitrogen balance. This may be interpreted as indicating that the nitrogen in these excess amino acids is not utilized under the conditions of this experiment. The drop in the nitrogen balance index for these groups approximately equals the index that may be calculated, assuming no utilization of the nitrogen in the excess amino acids fed. In Groups V and VI there is a significant rise in the nitrogen balance. The extra arginine fed to these 2 groups may be responsible for this rise. In these 2 groups, the nitrogen balance index calculated on the basis of no utilization of the amino acid nitrogen, is lower than that observed indicating, therefore, some usage of the excess amino acids. Previous studies indicate that excess arginine nitrogen added to casein is retained, in part, by the rat.[5] It should be pointed out that there appears to be a strain difference in the effects of excess methionine. For example, it has been found that 2.5% methionine, fed to Long-Evans strain rats, produced about the same effects in previous studies[4] as 4.8% produced in this one. Further work has shown that the addition of 7% methionine to the diet will produce negative nitrogen balance and tissue destruction in the Sherman strain rats.

The rats in all groups lost weight rapidly at first, and then more slowly during the first 6-8 days of the experiment (Table II). Thereafter, with the exception of those rats in Group II, a weight gain took place, so that at the end of 20 days the rats were nearly back to their original weights. The nitrogen balances paralleled the weight changes closely, being negative the first few days and even during the first collection period, but then positive for the last 12-14 days of the feeding. The rats receiving casein plus methionine (Group II), however, continued to lose weight throughout the 20 days, even though they were in positive nitrogen balance for the last 12 days.

In Fig. 1 is plotted the relationship between weight loss and nitrogen balance for the rats receiving casein alone (Group I). Fig. 2 illustrates the relationship between weight loss and nitrogen balance for Group II fed 12% casein plus 4.8% methionine. The loss in weight in the rats receiving excess methio-

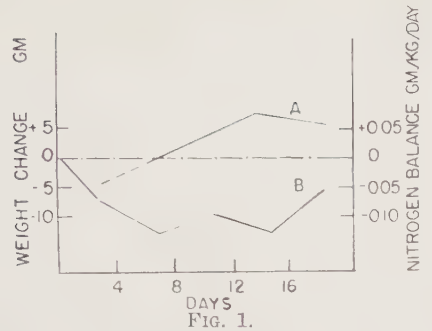

FIG. 1.

Relationship between weight loss and nitrogen balance for rats fed 12% casein.
Group I. Avg of 10 rats.
A. N balance B. Wt loss

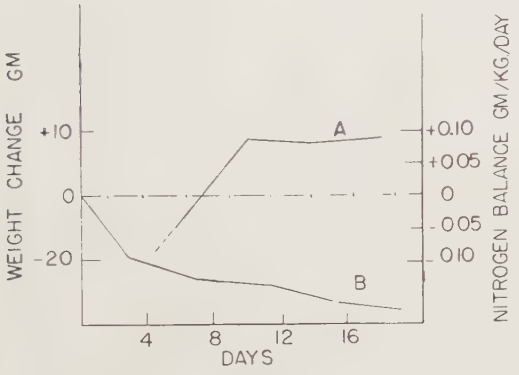

FIG. 2.

Relationship between weight loss and nitrogen balance for rats fed 12% casein + 4.8% *dl*-methionine.
Group II. Avg of 10 rats.
A. N balance B. Wt loss

nine while they were in positive nitrogen balance is associated with a loss in body fat. There is a marked lack of fat stores shown upon autopsy.[5]

The addition of glycine, or glycine plus arginine to the casein diet containing excess methionine counteracts the loss in weight caused by the excess methionine alone. Possibly glycine prevents the excessive lipotropic action of the methionine by aiding in its metabolism, or excretion. Based on previous studies,[5] larger amounts of methionine alone could cause continued loss in body nitrogen as well as fat, the animals never reaching positive nitrogen balance during the 20-day experimental period.

The excretions of creatine and creatinine are illustrated in Table II. The results are daily excretions and are averages of 20 determinations.

These data show that the addition of excess glycine to a casein diet does not significantly raise the creatinine or creatine excretion. The excretion of these materials is increased slightly by feeding excess methionine, alone, or in combination with glycine and arginine. Larger and more significant increases in the excretion of creatinine and creatine take place if more methionine is added to the diet since previous studies have demonstrated that excesses of methionine causing loss in body nitrogen will increase the excretion of creatinine.[5]

The effect of the diets on the organ weights is given in Table III.

The data in this table demonstrate that the addition of excess methionine to a casein diet causes an increase in liver and kidney weight and a slight increase in adrenal and thyroid weight; confirming previous results. The feeding of excess glycine causes an increase in liver weight but no other change. These data, together with those previously reported[5] demonstrate that both glycine and L-arginine antagonize in part the hypertrophic effect of the excess methionine.

That the increases in liver and kidney sizes were not due to increases in water content is demonstrated by the data in Table IV. The data in Table IV show that the addition of methionine, methionine plus glycine, or methionine plus arginine plus glycine, to a casein diet increases the nitrogen content of the kidneys. Glycine alone or in combination with arginine has no significant effect. Thus the hypertrophy of the kidney is associated with the presence of methionine.

Summary. In these experiments on rats, the addition of 4.8% glycine, 4.8% DL-methionine or 4.8% glycine plus 4.8% DL-methionine to a 12% casein diet did not alter the nitrogen balance as compared to controls receiving 12% casein alone but did reduce the nitrogen balance index, the excess amino nitrogen not contributing to the retention of nitrogen in the animal. The data indicates, on the other hand, that excess arginine (1.7%) contributes in part, to the retention of nitrogen. The addition of 4.8% glycine or

TABLE III.
Organ Weights of Rats Fed Various Nitrogen Sources.
Wet weights per 100 g of body weight. The results are averages obtained on 10 rats.

Group	Liver, g	Kidney, g	Adrenals, mg	Thyroid, mg	Testes, g	Seminal vesicles, g
I	2.66 ± .11	.617 ± .012	10.1	3.95 ± .26	.955	.310
II	3.15 ± .07	.856 ± .023	12.6	4.66 ± .19	1.07	.264
III	2.96 ± .05	.632 ± .014	8.8	4.01 ± .18	.912	.266
IV	2.91 ± .15	.707 ± .011	9.5	4.04 ± .07	.926	.312
V	2.79 ± .13	.788 ± .036	9.9	3.99 ± .10	.977	.228
VI	2.32 ± .11	.625 ± .016	9.1	3.88 ± .22	.960	.313

TABLE IV.
Water and Nitrogen Contents of Livers and Kidneys of Rats Fed Various Nitrogen Sources.
Averages on 10 rats.

	Water		Dry wt		Nitrogen		Total nitrogen	
Group	Liver %	Kidney %	Liver g/100 g B.W.	Kidney g/100 g B.W.	Liver %	Kidney %	Liver g/100 g B.W.	Kidney g/100 g B.W.
I	70.8	76.1	.815	.146	10.28	11.63	.084	.0169
II	70.9	76.4	.915	.201	10.23	11.61	.094	.0234
III	71.3	76.0	.846	.151	10.12	11.55	.086	.0174
IV	71.9	75.8	.794	.171	10.42	11.34	.083	.0194
V	70.3	76.9	.829	.181	10.53	11.16	.087	.0202
VI	70.7	76.0	.675	.149	10.86	11.29	.073	.0168

4.8% glycine plus 1.7% L-arginine to a casein diet containing excess methionine counteracted the weight loss and in part the kidney hypertrophy caused by the excess methionine. The slight increases in thyroid weights, associated with excess methionine, were also antagonized by glycine and arginine. Under the conditions of these experiments urinary creatinine and creatine excretion was not increased in rats by feeding excess glycine. The significance of these results is discussed.

16917

Therapeutic Effect of Choline Chloride in Dogs with Fat Emboli Produced by Bone Marrow Curettage.*

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Choline chloride as therapy for fat embolism was apparently used for the first time at Minneapolis General Hospital in 1948.

Moosnick, Schleicher,† and Peterson[1] observed that the fat content of human bone marrow is decreased 90% when a 1% choline chloride solution is given intravenously.

Methods. Twelve dogs were used to determine the value of choline chloride as a

* Supported by a research grant from the Graduate School, University of Minnesota.

† The authors wish to express their thanks to Dr. Emil Schleicher for offering the suggestion that choline might be of value in treatment of fat embolism and for his invaluable advice. They also express thanks to Dr. Steven Barron of Department of Pathology for his advice and assistance in preparation of microscopic material.

[1] Moosnick, F. B., Schleicher, E. M., and Peterson, W. E., *J. Clin. Invest.*, **1945**, **24**, 228.

therapeutic agent for fat embolism. Examinations of blood and urine were done to determine presence and size of fat globules.

Fat embolism was produced in 12 dogs according to Friesen *et al.*,[2] under pentobarbital anesthesia by doubly drilling through both cortices of the lower ⅓ of the humerus, and lacerating the bone marrow with a sharp curet. These dogs were observed for a period of 5 days, and then autopsied.

After surgery half the dogs were given .25 cc 1% choline chloride per kilogram body weight intravenously 3 times a day at 5 hour intervals into the leg vein opposite the traumatized humerus. The other half served as controls.

Blood specimens were collected daily from each dog.

Daily specimens of urine were collected by catheter. Five cc of urine was centrifuged for 5 minutes. One drop of urine from the meniscus was placed on a glass slide and one drop Sudan III was added. The preparation was then examined under high power after standing for five minutes. The size and number of fat globules were recorded.

Results. In the choline treated dogs, fat globules were found in the blood plasma between the second and fourth day and these varied from 4 to 12 micra. Three globules

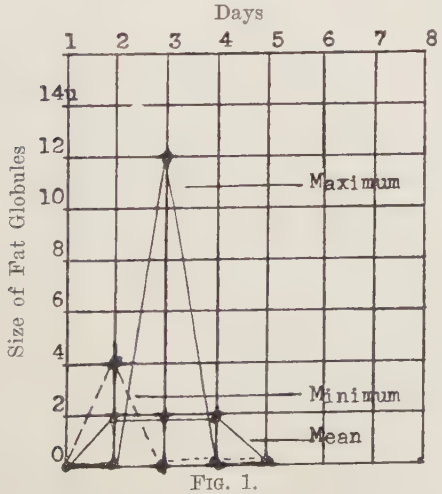

FIG. 1.
Fat in Blood of Choline Dogs.

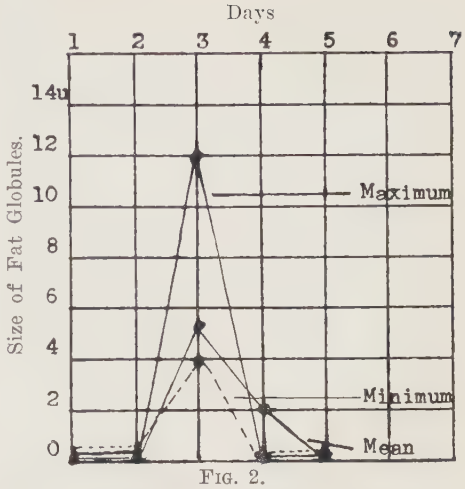

FIG. 2.
Fat in Urine of Choline Dogs.

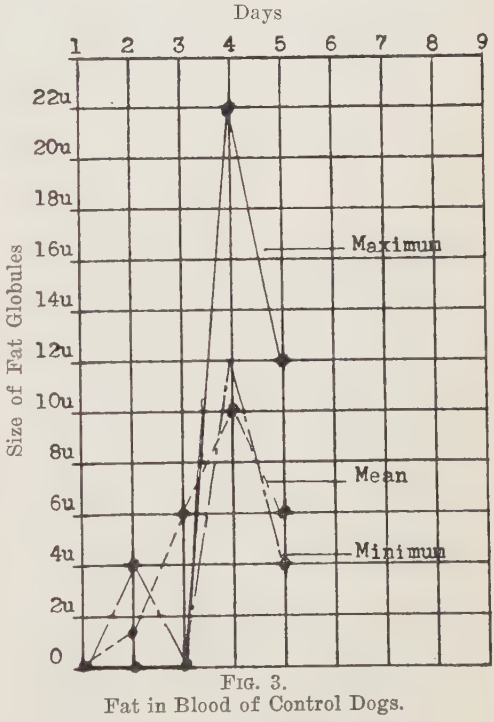

FIG. 3.
Fat in Blood of Control Dogs.

were the maximum per field. None was observed in blood plasma on the fifth day.

Fat globules appeared in the urine of the dogs treated with choline on the third and fourth day after surgery, varying from 4 to 12 micra in diameter. but not more than 2 fat globules per field were noted. None was seen in the urine on the fifth day.

2 Friesen, S. R., Merendino, A. K., Baronofsky, Ivan, Mears, Frederick B., and Wangensteen, O. H., *Surg.*, 1948, **24**, 148.

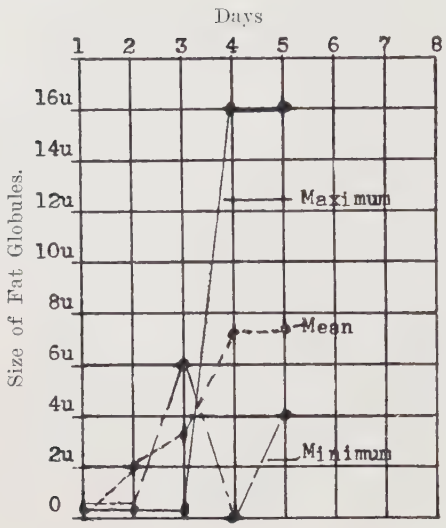

FIG. 4.
Fat in Urine of Control Dogs.

In the control dogs, fat globules were present in the urine and blood plasma from 2nd day after surgery to the time of autopsy. Maximum fat was present from the third day on. The size of fat globules varied from 4 to 20 micra; many were seen per high power field.

After autopsy, formalin fixed, hematoxylin and eosin stained sections of lungs showed fat emboli in all dogs with no appreciable difference between treated animals and controls.

Conclusion. Choline appears on the basis of plasma and urine examinations to have been of value in the management of experimentally produced fat embolism in dogs. Lung sections at five days after trauma proved inconclusive.

Note. Choline has been used in clinical cases of fat embolism with apparently very striking results. This is to be reported.

16918 P

Acute Occlusion by Ligature of the Portal Vein in the *Macacus rhesus* Monkey.*

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It is commonly accepted today that sudden occlusion of the portal vein both in the human and in animals is followed within a matter of minutes to hours by death. The first demonstration of this phenomenon is credited to Oré,[1] who noted that rabbits in which the portal vein was ligated survived but a short time. Since this original observation a large number of confirmatory reports can readily be found. The most definitive study is that of Elman and Cole,[3] who proved not only that ligature of the portal vein in dogs and cats is followed promptly by the death of the animal but also that failure to survive the procedure was due to loss of effective circulating blood volume secondary to pooling within the obstructed splanchnic venous bed. In addition to animal experiments such as these, there are a number of reports in the literature of occlusion of the portal vein in the human by thrombosis, either septic or bland, and by ligature made necessary either in the course of a surgical operation or secondary to intra-abdominal trauma.

The conclusion drawn from the animal experiments is that sudden obstruction of the portal vein is incompatible with life; from a review of the phenomenon as it is reported in the human the implication is also that in general a patient cannot survive sudden portal occlusion. A few reports are available for study, however, which indicate that occasionally the so-called "hepatopetal" veins may be sufficiently well developed to take over im-

* Supported by U. S. Public Health Service grant.

1 Quoted by Neuhof.[2]

2 Neuhof, Harold, *S. G. and O.*, 1913, **16**, 481.

3 Elman, R., and Cole, W. H., *Arch. Surg.*, 1934, **28**, 1166.

mediately the burden of returning sufficient portal blood to the systemic circulation to prevent shock and death.[4] As far as can be determined, however, it has never been conclusively settled whether or not the human can survive sudden occlusion of the portal vein by ligature. Because occasionally a patient with pancreaticoduodenal cancer must be refused radical resection because of invasion of the portal or superior mesenteric veins, it has recently become important to decide whether or not resection of these structures is compatible with life and prolonged survival. Today it is generally accepted that whatever else is done during radical pancreaticoduodenectomy the portal and superior mesenteric veins must preserved.

In an effort to elucidate this problem further, its study has been undertaken in the *Macacus rhesus* monkey. This animal was selected because the anatomy of the portal, superior mesenteric, and splenic veins is nearly identical with that of the human. Furthermore, the anatomical relationships of the pancreas and duodenum approximate the retroperitoneal position of these structures found in man and not present in dogs, cats, rabbits, etc.

Experimental Procedure. Adult *Macacus rhesus* monkeys were operated upon under open drop ether anesthesia and various segments of the portal circulation occluded by double ligature with medium silk. The animals recovered promptly from anesthesia and, except for the discomfort apparently due to the celiotomy wound, gave no evidence of having been disturbed by the procedure. They are all surviving, the postoperative period varying from 16 days to 6 weeks. In an effort to document further these experiments, all of the animals have been subjected to portal venography with 2-4 cc of thorium dioxide injected into mesenteric venous channels at about the level of the mid-jejunum. All of the venograms (for representative venograms see Plates I and II) have demonstrated complete occlusion of the portal vein, blood gaining access to the systemic circulation by various routes, chiefly, however, the right and left colic veins and the small retroperitoneal vessels in the region of the head of the pan-

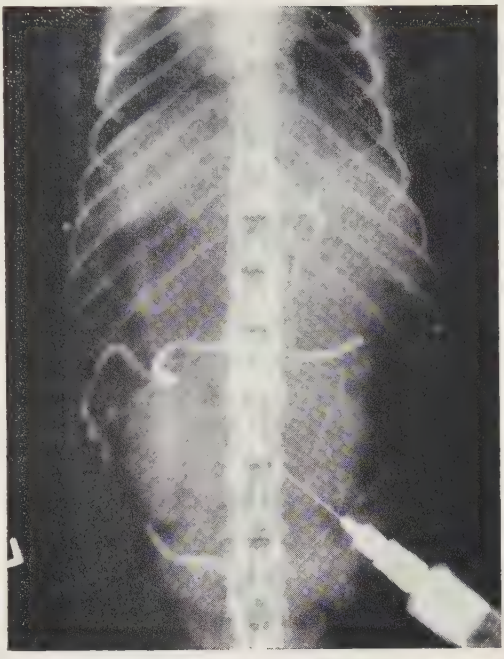

PLATE 1.
Experiment No. 4. Splanchnic venogram (3 cc thorium dioxide) 13 days following ligation of the superior mesenteric vein.

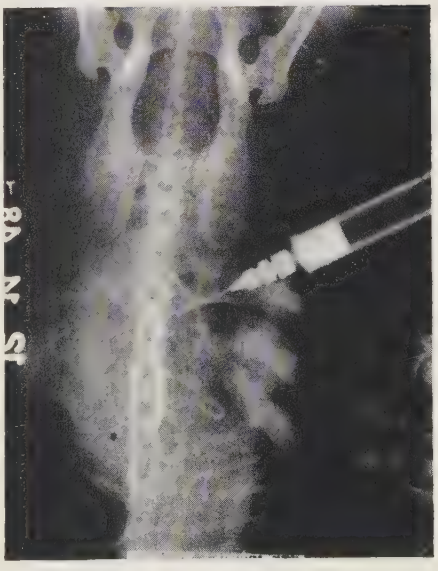

PLATE 2.
Experiment No. 6. Splanchnic venogram (3 cc thorium dioxide) 6 days following ligation of portal, superior mesenteric, and splenic veins.

[4] Colp, Ralph, *S. G. and O.*, 1926, **43**, 627.

TABLE I.
Acute Occlusion by Ligature of the Portal Vein in the *Macacus rhesus* Monkey.*
All recovered and present condition is normal.

Exper. No.	Vessels ligated	Sex	Days post-op.	Major collateral circulation
1	Portal vein	F	51	Splenic vein Inferior mesenteric vein Middle colic vein
2	" "	M	50	Same
3	" "	M	49	Same
4	Superior mesenteric vein	F	43	Middle colic vein Retroperitoneal vein Hepatic ligament vein
5	Portal vein Superior mesenteric vein	F	38	Middle colic vein Inferior mesenteric vein
6	Portal vein Superior mesenteric vein Splenic vein	F	23	Retroperitoneal vein Middle colic vein Omental vein
7	Same	F	16	Same

* Seven *Macacus rhesus* monkeys subjected to ligation of various segments of the portal venous system. Survival periods and chief systemic pathways of anastomosis are indicated in the column headed "Major Collateral Circulation."

creas. To date none of the animals has developed clinically detectable nutritional deficiencies or ascites. Table I outlines the seven experiments performed.

Summary and conclusions. Various segments of the portal circulation (including the portal vein) have been suddenly occluded by ligature in 7 adult *Macacus rhesus* monkeys. All of the animals have survived this procedure uneventfully and are in apparent good health from 16 to 51 days postoperatively. By means of portal venography anastomotic channels have been demonstrated by way of which blood is immediately returned to the systemic circulation in sufficient quantities to prevent these animals from succumbing to shock due to depletion of their circulating blood volume.

16919

The Ulcer-Inhibiting Action of Pyrogens.

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A series of urinary extracts under investigation for their ulcer-inhibiting action in the Shay rat, were found to contain a high concentration of pyrogens. Since Necheles[1] had shown that pyrogens suppressed gastric motility in dogs, it seemed desirable to study the inhibitory effect of purified pyrogens in the pylorus-ligated rat. Pyrogens prepared from cultures of *B. prodigiosus, Pseudomonas aeruginosa* and *E. typhi* were investigated.

Method. The assay procedure for estimating anti-ulcer activity was essentially that of Pauls, Wick, and MacKay.[2] Sprague-Dawley male rats of 150-170 g weight were fasted for 48 hours in cages with coarse mesh bottoms. Water was given *ad libitum*. After ether anesthesia, the duodenum was exposed, grasped lightly with fine forceps and ligated at the pyloric sphincter with braided silk thread. The incision was closed with metal clips and painted with collodion solution in order to avoid ingestion of blood residue from the wound.

[1] Necheles, H., *Am. J. Physiol.*, 1942, **137**, 28.

[2] Pauls, F., Wick, A. N., and MacKay, E. M., *Gastroenterology*, 1947, **8**, 774.

TABLE I.

Pyrogen	M.P.D.,* μg per kg	Nitrogen, %	Reducing substances, %	Glycosamine, %
B. prodigiosus	.005	.7	68.9	2.5
Pseudomonas aerugenosa	.02	1.1	64.5	4.2
E. typhi	.01	.8	58.7	3.1

* M.P.D. or minimal pyrogen dose refers to the minimal amount which will cause a temperature rise of 0.6°C in rabbits (U.S.P. XIII).

TABLE II.

Preparation	Dose range, μg	Route	Total No. rats	No. ulcerated	Ulcer index
Controls	—	Intrav.	56	53	315
Prodigiosus pyrogen	3-10	”	28	10	72
” ”	15-20	”	24	7	55
” ”	25-30	”	30	5	31
” ”	35-50	”	20	1	15
” ”	10-25	Intraper.	15	8	140
” ”	50	”	10	2	80
” ”	100-1000	”	15	0	0
” ”	100-1000	Oral	3	3	367
Pseudomonas pyrogen	10-50	Intrav.	29	8	52
” ”	100-500	”	11	0	0
Typhoid pyrogen	50-250	”	8	2	25

Pyrogens were dissolved in pyrogen-free dilute buffer and injected in 0.5 ml volumes intravenously in a tail vein or intraperitoneally just prior to pyloric ligation. In one experiment, pyrogen solution was given orally by stomach tube a few minutes following the operation when recovery from anesthesia was nearly complete. Control rats received 0.5 ml pyrogen-free buffer solution intravenously. Animals were sacrificed at the end of eight hours. Volume and free acidity of stomach contents were measured and recorded and the degree of ulceration scored using an arbitrary scale of +4 to +1 according to the number and size of lesions observed. Zero was recorded when no macroscopic abnormality was seen. The average score for each group was multiplied by 100 to give an "ulcer index" following the method of Risley, Raymond, and Barnes.[3]

Pyrogens. Pyrogens were prepared from pure cultures grown on synthetic medium[4] and isolated by a procedure to be described in a subsequent publication. Full analytical data on these pyrogens will appear later.[5] However, Table I gives the pertinent information on each of these highly purified preparations used in the experiments reported here.

Results. Experimental results are recorded in Table II which for brevity is tabulated in dose ranges of administered pyrogens rather than for separate dosages.

Among 56 control rats not treated with pyrogen, 53 showed ulceration at the end of 8 hours to an average degree of +3.15 or an ulcer index of 315. Intravenous doses of 3-10 μg prodigiosus pyrogen protected 18 of 28 rats and reduced the ulcer index to 72. Increasing doses increased the number of rats protected and reduced the ulcer index to a low of 15 at 35-50 μg doses. Intraperitoneally, corresponding amounts of this pyrogen are somewhat less effective than when given intravenously. However, doses ranging from 100-1000 μg offer complete protection, none of the 15 rats treated with this amount of pyrogen showing any ulceration. Orally this pyrogen appears to be completely lacking in protective activity against ulceration. The data, however, are too limited to allow gen-

[3] Risley, E. A., Raymond, W. B., and Barnes, R. H., *Am. J. Physiol.*, 1947, **150**, 754.

[4] Rodney, G., and Welcke, M., *J. Bact.*, 1945, **50**, 129.

[5] Rodney, G., and Devlin, H. B., to be published.

eralization with respect to this mode of administration.

Pseudomonas pyrogen was used in only 2 dose ranges of 10-50 μg in 29 rats and 100-500 μg in 11 rats. Critical examination of the individual protocols indicates that this pyrogen had slightly less inhibitory effect on ulceration than the prodigiosus pyrogen which correlated with the comparatively low pyrogenic activity as measured in the rabbit test.

Typhoid pyrogen was used only in the larger dose range, two of eight rats showing minor ulceration with this preparation.

Not shown in the table but deserving of mention are results with a non-pyrogenic polysaccharide prepared by the pyrogen isolation technic from pneumococcus type II cultures. This substance showed no anti-ulcer activity in 4 rats in doses up to 200 μg.

Also omitted are detailed data on gastric volume and free acidity. However, it may be stated that among all rats studied in these and related experiments, the mean gastric volume of 208 ulcerated rats was 8.9 ± 2.6 (S.D.) ml whereas in 94 non-ulcerated rats secretion amounted to 5.9 ± 3.2 ml. Likewise free acidity of gastric contents from ulcerated rats was equivalent to 5.3 ± 2.4 ml N/10 NaOH. Non-ulcerated rats showed values of 3.0 ± 2.3 ml. Among both groups of data the differences between the means from ulcerated and non-ulcerated rats are statistically significant.

Conclusion. As judged by response in the Shay rat, pyrogens from *B. prodigiosus, Pseudomonas aerugenosa* and *E. typhi* are highly active ulcer-inhibiting substances. Whether the presence of these substances in various tissue and urinary extracts may account for the reported anti-ulcer activity of these preparations remains to be investigated. The mode of action of pyrogens is not clear but may be associated with the obviously diminished acidity and volume of gastric secretion which occurs as a result of administration of these substances. There was observed no evidence, however, of any general "toxicity" in the animals which received purified pyrogens.

Summary. Intravenously administered purified pyrogens from cultures of *B. prodigiosus, Pseudomonas aeruginosa* and *E. typhi* possessed marked ulcer-inhibiting action in the Shay rat.

16920

Increased Requirement for Pteroyl Glutamic Acid During Lactation.*

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A series of experiments had been undertaken in an attempt to produce an anemia in fetal and newborn rats. One of the technics employed was the feeding of succinyl sulfathiazole (SS) to the maternal rats. During the course of the study, it was observed that the newborn rats from mothers receiving the diet containing SS, died within five days after birth. It was thought that this high mortality among the newborn might be due to either a toxic effect or to a failure of lactation.

Preliminary experiments indicated that SS or its hydrolysis products did not have any toxic effect on the newborn rats. Although SS passes through the alimentary tract substantially unchanged and is not absorbed, some small part of it is hydrolyzed to sulfathiazole which may be absorbed. On a diet containing 1% SS, rats were found to have a blood level of approximately 0.3 mg % sulfathiazole.[1] The administration of SS or

* This work was supported in part by a grant from the Foundation for Vision for the study of Retrolental Fibroplasia.

TABLE I.
Effect of Diet of Pregnant Rats on the Survival Time of the Newborn.

Diet	No. of litters	No. of newborn	Birth wt, g	Average survival, days	No. of newborn surviving 20 days	% mortality
Control	4	30	5.5	18.1	26	13
SS diet	12	94	5.3	2.2	0	100
SS " + liver	5	40	5.1	4.1	0	100
SS " + alfalfa	4	32	5.8	15.2	23	28

TABLE II.
Survival of Newborn Rats from Mothers on a High Pteroylglutamic Acid Diet.

Diet	No. of litters	No. of newborn	Birth wt, g	% of newborn surviving 20 days
Control	6	42	5.1	97
SS diet	8	56	5.2	91
SS " + alfalfa	8	55	5.2	96

sulfathiazole intragastrically, or intraperitoneally, at a level estimated to be about 10 times that ingested in the milk was without effect on the newborn rats from mothers on a normal diet. When small amounts of sulfathiazole (0.08%) were included in the diet, there was no effect on lactation. Since this dietary level of sulfathiazole produced about the same level of blood sulfathiazole as did 1% SS, the lactational failure could not be ascribed to a direct toxicity of SS or sulfathiazole.

A further experiment indicated that the fatality among the newborn rats from mothers receiving the diet containing SS was most probably the result of inanition. When newborn rats (12-36 hours after birth) from mothers on a normal diet were substituted for the newborn litter of a mother on a diet containing 1% SS, the normal newborn died within a few days. On the other hand, the newborn from mothers on the SS diet were successfully raised to weaning by mothers receiving a normal diet.

Since a toxic factor did not appear to be responsible, it was considered that the failure in lactation might be due to the need for some essential nutrient, normally synthesized by the intestinal flora, but now lacking because of bacterial suppression by the SS.

Experimental. In all the following experiments, female rats were placed on the control or experimental diets 10 days before mating. The females were kept on their respective diets until 20 days after the birth of their young. In the first series of experiments (Table I), the litters were limited to 8 young; in the second (Table II), to 7 young.

The control diet consisted of 28 g vitamin-free casein (SMACo), 56 g carbohydrate (sucrose or cerelose), 10 g lard, 2 g corn oil, 4 g salt mixture,[2] 1.0 mg thiamine HCl, 1.5 mg riboflavin, 1.0 mg pyridoxine HCl, 10 mg nicotinic acid amide, 4.0 mg calcium pantothenate, 100 mg inositol, 100 mg choline chloride, 100 mg p. aminobenzoic acid, 0.5 mg 2-methyl naphthoquinone, 0.05 mg biotin, 0.02 mg pteroylglutamic acid,† 2.0 mg α-tocopherol, 2500 IU vitamin A, and 360 IU vitamin D. The experimental diet (SS diet) was the same except that 1 g of succinyl sulfathiazole was substituted for an equal amount of carbohydrate. The diets were fed *ad libitum*.

A comparison was made of the effect of the control and SS diets on lactation. The survival time of the newborn rats was taken as a measure of lactation. Although some of the litters of newborn from the SS diet group had milk in their stomachs the first and second days, they were all dead by the fifth day

[1] Williamson, M. B., Abstr., *Div. Biol. Chem., Am. Chem. Soc.*, 1948, 55C.

[2] Hubbel, R., Mendel, L., and Wakeman, D., *J. Nutrition*, 1937, **14**, 237.

† The pteroylglutamic acid was generously supplied by Dr. M. C. Lockhart of the Lederle Laboratories.

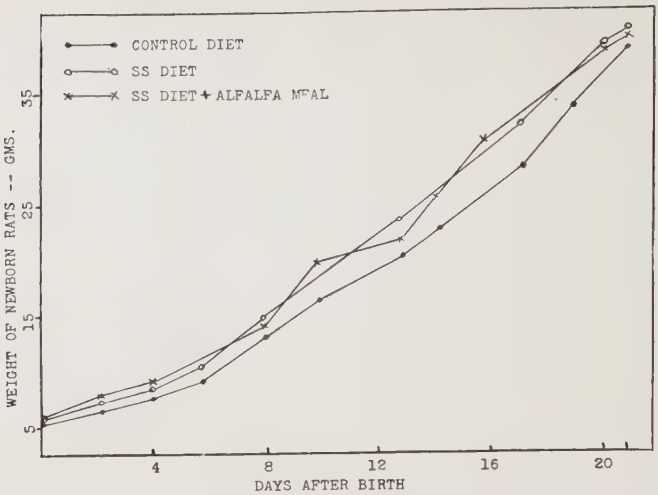

FIG. 1.

The rate of growth of newborn rats from mothers on a synthetic diet containing pteroylglutamic acid.

after birth; in no case was milk observed after the second day. This might be taken to indicate that in some cases, the crucial nutritional factor required to maintain lactation was not depleted until after one or two days of lactation. After the depletion, lactation failed with the consequent death of the newborn rats. Only 13% of the control newborn died before 20 days; it seems highly improbable that this mortality was due to lactational failure.

Further experiments in which supplements were added to the SS diet were undertaken. When 1% dry defatted liver powder was added to the SS diet, a slight but questionable increase in survival time was noted. In another experiment, 10% alfalfa meal was used. The casein content of the SS diet containing alfalfa meal was adjusted so that the total nitrogen content was the same as that of the control diet. The alfalfa meal supplement markedly improved the SS diet so that it was almost as efficient as the control diet in supporting lactation. The results of these experiments are shown in Table I.

Since it is known that added nutritional "stress" occurs during periods of pregnancy and lactation,[3] it was thought that there might possibly be some nutrient which was required at an even higher level than was supplied by the preceding diets. Richardson and Hogan[4] have reported the successful rearing of rat litters by mothers on a diet essentially the same as the control diet, except that it contained no pteroylglutamic acid or SS. The presence of SS in the diet, however, makes the necessity for exogenous pteroylglutamic acid crucial.[5] Therefore, the effect of a higher level of pteroylglutamic acid on lactation was investigated.

The control diet, the SS diet and the SS + alfalfa meal diet were supplemented to contain 1.0 mg of pteroylglutamic acid per 100 g of diet. Almost all the newborn rats from all groups survived until 20 days after birth. The results are indicated in Table II. The rates of growth of the newborn are shown in Fig. 1.

It can be seen that all the newborn grew at approximately the same rate, and hence, most likely received about the same amount of milk. It then follows that the level of pteroylglutamic acid in the maternal diet was at, or above, the optimal level for the support of lactation. Recent reports have shown the beneficial effects of pteroylglutamic acid on lactation.[6,7]

[3] Ershoff, B. H., *Physiol. Rev.*, 1948, **28**, 107.

[4] Spicer, S. S., Daft, F. S., Sebrell, W. H., and Ashburn, L. L., *Pub. Health Rep., U.S.P.H.S.*, 1942, **57**, 1559.

[5] Richardson, L. R., and Hogan, A. G., *J. Nutrition*, 1946, **32**, 459.

It is interesting to note that the maternal requirement for pteroylglutamic acid appears to be very much greater during the period of lactation than during pregnancy. This is supported by the fact that the birth weights of all the newborn were found to be about the same (5.5 ± 0.8 g) regardless of the maternal intake of pteroylglutamic acid.

Summary. The inclusion of succinyl sulfathiazole in the diet of maternal rats during pregnancy depresses lactation. Relatively high levels of pteroylglutamic acid are required to maintain lactation. Higher levels of pteroylglutamic acid appear to be required during lactation than are required during pregnancy.

6 Nelson, M. M., and Evans, H. M., *Arch. Biochem.*, 1947, **13**, 265; 1948, **18**, 153.

7 Sica, A. J., Allgeier, A. M., and Cerecedo, L. R., *Arch. Biochem.*, 1948, **18**, 119.

16921

Acetone-Ether Extracted Antigens for Complement Fixation with Certain Neurotropic Viruses.

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A method is described for the preparation of antigens for complement-fixation tests with certain neurotropic viruses. This antigen has been found to be practical, nonanticomplementary, specific and reliable. It is furthermore, easily prepared with ordinary laboratory equipment and can be made available, if necessary, within 6 hours from the time when the tissue is harvested, thus being useful for field work. Since the preparation depends on the elimination of the acetone-ether soluble fraction of the brain tissue with considerable reduction of lipid, it has the advantage of doing away with nonspecific reactions with Wassermann-positive human sera.[1,2]

Method of preparation. Brain tissue from mice infected intracerebrally with the desired virus is harvested; preliminary bleeding of the mice[2] is advisable in order to reduce the amount of hemoglobin present in the final product. The weighed tissue with 20 volumes of acetone is placed in a Waring blendor and allowed to run for 2 minutes. The blendor used for this purpose is surrounded by a metallic jacket which is filled with ice and water to maintain a low temperature; less preferably, the extraction can be carried out in the absence of this device. The suspension is then poured into a 250 ml bottle and centrifuged at 1500 rpm for 1 minute; the supernate is discarded. All the foregoing procedures should be rapidly performed. To the sediment is added 20 volumes (referred to the initial weight of the wet-brain tissue) of fresh acetone; the bottle is closed with a cork stopper, shaken by hand at intervals and kept at room temperature for 20 minutes. The preparation is then centrifuged as before, the supernate which is slightly opalescent, as are the subsequent supernates, is discarded. To the sediment is added 20 volumes of a mixture of equal parts of acetone and anhydrous ethyl ether. Again the suspension is kept at room temperature for 20 minutes, shaken occasionally by hand, centrifuged at 1500 rpm for 1 minute and the supernate discarded. The sediment is washed twice in succession with 20 volumes of ethyl ether each time and held for 20 minutes at room temperature. After the last ether extraction and centrifugation, the sediment is dried by connecting the centrifuge bottle with a vacuum oil pump; in a short time, from 15 minutes to 1 hour depending on the amount involved, the residual ether is evaporated and the sediment remains as a

1 DeBoer, C. J., and Cox, H. R., *J. Bact.*, 1946, **51**, 613; *J. Immunol.*, 1947, **55**, 193.

2 Espana, C., and Hammon, W. D., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 101.

TABLE I.
Protein and Lipid Contents of Complement-fixing Antigens.

Antigen	Method of preparation	Diluent/original brain tissue	Protein, mg/ml	Lipid, mg/ml
Japanese B	Acetone-ether extraction	3:1	10.7	0.18
Normal brain		1:1	38.8	1.33
" "		5:1	4.5	0.37
Japanese B	No extraction with acetone-ether; frozen and thawed	10:1	1.2	0.32
Normal brain		10:1	2.0	0.30

dry, fine powder. It is necessary to set a trap preferably containing cotton soaked in heavy oil between the bottle containing the material and the pump, owing to the fact that the evaporation proceeds rapidly and some of the fine powder may be drawn into the pump. After drying, the material always kept in the same bottle is resuspended in physiological saline solution; the amount of the latter can vary between 2 and 5 times the weight of the initial brain tissue, according to the concentration desired; ordinarily, 3 volumes of saline suffice. The suspension is placed in the refrigerator overnight; almost equally good results are, however, obtained if it is kept at room temperature for 30 minutes. The suspension is centrifuged in the angle-head centrifuge at 10,000 rpm for 1 hour; the supernate is pipetted off, Merthiolate in final concentration of 1:10,000 added and it is then stored in a glass-stoppered bottle at 2°C. This extract constitutes the antigen.

Complement-fixing antigens have been prepared by this means with the following viruses: Japanese B, St. Louis, Western equine, and Russian Far East encephalitis. No attempt has been made to lyophilize the preparations; in liquid form they can be kept at least 4 months at 2°C. It should be borne in mind that the antigens contain active virus immediately after preparation; it may be possible to inactivate it by formolization or ultraviolet radiation. The writer, however, employs the material without the inactivating treatment.

The general procedure for carrying out the complement-fixation test and for preparation of animal immune sera has been described elsewhere.[3] Unless otherwise indicated the acetone-ether antigens used in the following experiments had been prepared by resuspending the final material in 3 volumes of diluent, referred to the initial wet-brain tissue weight.

Protein and lipid content. Although the precise chemical nature of the antigens is unknown, it was observed that the titer of samples deriving from the same lot increased with the concentration of protein in solution. When lipids were not extracted, increased protein concentration in the antigen resulted in increased lipid in suspension, thus yielding anticomplementary and nonspecific effects. When antigens were prepared by the present method, the concentration of protein could be increased considerably, with resulting higher titer, and lipids, although not wholly removed, were present in such small amount as to give no anticomplementary reaction. Table I shows the result of determinations of protein and lipid in antigens prepared by the present, as well as in antigens prepared by a previously described method,[4] without lipid extraction.*

It can be seen that the ratio of protein to lipid which was 7 to 1 or less in the nonextracted antigens, was 60 to 1 in some of the acetone-ether treated antigens. Furthermore, the concentration of protein could be increased in the latter to a value far beyond that shown by the nonextracted antigens, with no indication of anticomplementary effect.

Wassermann-positive sera. An investigation of the possible nonspecific reaction with Wassermann-positive human sera† revealed that under present conditions of inactivation

[3] Casals, J., *J. Immunol.*, 1947, **56**, 337.

[4] Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

* Protein was estimated by quantitative biuret test. Lipid determinations were made by Dr. E. H. Ahrens, Jr., of the Rockefeller Institute Hospital, to whom many thanks are due.

TABLE II. Complement-fixation Reaction with Wassermann-positive Human Sera Inactivated at 63°C for 3 Minutes and Acetone-ether Extracted, and Nonextracted Antigens.

Serum dilutions Serum No.	Acetone-ether extracted antigen								Nonextracted, frozen and thawed antigen								No antigen, saline	
	West. equine				Japanese B				Japanese B				St. Louis					
	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:32	1:2	1:4	1:8	1:16	1:32	1:2	1:4
1	0	0	0	0	0	0	0	0	4	4	3	±	0						0	0
2	0	0	0	0	0	0	0	0	3	2	1	0	0						0	0
3	0	0	0	0	0	0	0	0	2	1	0	0	0						0	0
4	2	0	0	0	3	2	0	0	4	4	3	±	0						0	0
5	0	0	0	0	0	0	0	0	4	3	±	0	0						0	0
6	0	0	0	0	0	0	0	0	3	3	3	±	0						0	0
7	0	0	0	0	0	0	0	0						0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0						4	4	0	0	0	0	0
9	0	0	0	0	0	0	0	0						0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0						3	3	±	0	0	0	0
11	0	0	0	0	0	0	0	0						2	±	0	0	0	0	0
12	2	0	0	0	2	0	0	0						4	4	1	±	0	2	0

Degree of fixation is expressed from 0 indicating no fixation to 4 indicating complete fixation.

(63°C for 3 minutes) they reacted nonspecifically with antigens of the former type, but not generally with those acetone-ether treated (Table II).

Anticomplementary effect. Numerous titrations of the anticomplementary effect of acetone-ether extracted antigens have been carried out under test conditions, namely incubation of the mixture of antigen and complement for 18 hours at 2-4°C. Table III shows the result of the titrations of complement in 3 different tests, in the presence of extracted antigens and of saline. When the antigens were prepared by resuspending the extracted brain tissue in 2 or more volumes of diluent (referred to the initial brain weight), the titer of complement was the same whether antigen was present or not. The concentrations of protein and lipid in these antigens were of the order of 20 mg or less, and 0.6 mg or less, per ml, respectively. On the other hand, when 1 volume of diluent or less was used, the resulting antigens were somewhat anticomplementary and gave a degree of nonspecific cross-reaction.

Specificity of the Reaction. The specificity of the complement-fixation reaction with acetone-ether extracted antigens was investigated mainly with mouse hyperimmune sera, and in a few cases, with human convalescent sera. Serial twofold dilutions of serum beginning with dilution 1:2 were tested against several antigens, either undiluted or diluted as indicated. The high dilutions of serum served to indicate that high titers of antibody could be obtained with these antigens, whereas the low dilutions showed that no cross-reactions occurred between different viruses, except in those cases when they are known to exist naturally, as for instance, between Japanese and St. Louis encephalitis viruses.[5] Examples of such tests are given in Table IV, in which are noted the high titers and the specificity of the antigens.

Titer of Antigens. When the extracted

† Wassermann-positive sera were obtained through the courtesy of Miss J. Haber, New York Hospital, New York. When received these sera had already been inactivated by heating at 63°C for 3 minutes.

[5] Casals, J., *J. Exp. Med.*, 1944, **79**, 341.

TABLE III.
Titration of Anticomplementary Power of Acetone-ether Extracted Antigens.

Test No.	Antigen	Diluent/original brain tissue	Protein, mg/ml	Complement: diluted fresh guinea pig serum 1:30 Amount of complement in ml .16	.14	.12	.10	.08	.07	.06	.05	.04
1	None, saline			0	0	0	0	0	0	±	2	3
	Normal brain	2:1	16.0	0	0	0	0	0	0	±	2	3
	Japanese B	1:1	30.0	0	0	0	0	±	2	2	3	4
	West. equine	3:1	9.0	0	0	0	0	0	0	1	2	2
2	None, saline				0	0	0	0	±	1	2	
	Normal brain	3:1	10.9		0	0	0	0	0	1	2	
	Japanese B	3:1	11.7		0	0	0	0	±	1	3	
3	None, saline			0	0	0	0	1	2	2		
	Normal brain	0.9:1	45.0	0	0	2	3	4	4	4		
	Japanese B	3:1	11.7	0	0	0	0	1	2	3		

TABLE IV.
Complement-fixation Tests with Acetone-ether Extracted Antigens; Mouse Hyperimmune and Human Convalescent Sera.

Antigen Type	Protein, mg/ml	Dil.	Serum Species	Japanese B	Russian Far East	Western equine
Japanese B	9.7	1:4	Mouse	1:128*	0†	0
Russian Far East	8.5	1:4		0	1:64	0
West. equine	9.0	1:4		0	0	1:8
Japanese B	30.6	Undil.	Mouse	1:32		0
West. equine	7.9	”		0		1:8
Normal brain	17.5	”		0		0
			Human, A‡	1:512		
Japanese B	9.7	”	” B	1:256		
			” A	0		
West. equine	9.0	”	” B	0		

* Highest dilution of serum giving a 2+, or better, reaction. The first tube had in all cases serum dilution 1:2.

† O indicates that no fixation occurred in any tube.

‡ The 2 human sera in this test were derived from individuals convalescent from Japanese B encephalitis.

brain tissue was resuspended in 3 volumes of diluent, the titer of the antigens was between 1:16 and 1:128, depending on several factors, such as the type of virus, the particular lot of brain tissue, and the relative amounts of serum and antigen that were employed. With respect to the latter it should be noted that, unlike what happens with nonextracted antigens,[3] the titers of both serum and antigen depend on their dilutions; more so does this apply to serum of which the titer is closely correlated with the number of units of antigen (Table V). This observation confirms that of Espana and Hammon,[2] who observed a similar phenomenon with their benzene-extracted antigens. Thus if a “box” titration were performed, with dilutions of serum tested against dilutions of antigen, results as given in Table V could, as a rule, be found.

It can be seen in Table V that the titer of the serum varied from 1:32 to 1:128 depending on whether antigen was used undiluted or in dilution 1:16. Conversely, the titer of the antigen was 1:64 when serum was used in dilution 1:2 and 1:128 with serum in dilution 1:16. In order to secure the highest titer for a given serum it seems advisable to use, as recommended by Espana and Hammon,[2] from 8 to 16 units of antigen. One unit of antigen is the amount contained

TABLE V.

Complement fixation Test with Japanese B Encephalitis Virus Antigen and Mouse Hyperimmune Serum. Acetone-ether Extracted Antigens.

Antigen		Dilutions of serum							
Type	Dil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Japanese B	1:1	4	4	4	4	3	0	0	0
	1:2	4	4	4	4	4	0	0	0
	1:4	4	4	4	4	4	2	0	0
	1:8	4	4	4	4	4	4	±	0
	1:16	4	4	4	4	4	4	2	0
	1:32	4	4	4	4	4	4	2	0
	1:64	3	3	4	4	4	3	1	0
	1:128	0	±	1	2	1	±	0	0
	1:256	0	0	0	0	0	0	0	0
West. equine	1:1	0	0						

in the highest dilution of antigen which in a box titration gives a 2+ or better reaction; in the example shown, dilution of antigen 1:128 equals one unit.

To conclude, reliable, high-titered antigens for complement-fixation tests with certain neurotropic viruses have been prepared by a simple method of extraction at room temperature of infected brain tissue with acetone and ethyl ether.

16922

Intravenous Infusions of a Combined Fat Emulsion into Human Subjects.*

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In previous papers from this laboratory, an emulsion of fat combined with protein and glucose was reported to have been administered intravenously into dogs with safety and with nutritional benefits.[1,2] The overall advantages of this intravenous emulsion were such that it warranted the present clinical investigation of its effects on human subjects. This however was not done without precedent for Holt had pioneered studies on intravenous fat in a series of pediatric patients whilst others reported sporadic attempts in adult humans.[3,4] The present report is devoted to the method of preparation of the combined fat emulsion and the effects of its intravenous infusion on human subjects.

Preparation of the Combined Fat Emulsion. The successful preparation of this emulsion is dependent upon adequate and thorough homogenization and emulsification which is accomplished in a specially constructed homogenizer adapted to medical usage. Basically the mechanism is that of a high pressure pump which forces the fluid mixture through a minute orifice against the resistance of a strong steel springed valve. The machine consists of three component parts: a reservoir tank with a high speed agitator incorporated

* Aided by grants from the Ben Lewis Fund in Experimental Surgery. Clinical investigation done at Goldwater Memorial Hospital (New York University, Third Surgical Division).

1 Shafiroff, B. G. P., and Frank, C., *Science*, 1947, **106**, 474.

2 Shafiroff, B. G. P., Baron, H. C., and Roth, E., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 387.

3 Holt, E., Jr., Tidwell, H. C., and Scott, T. F. McNair, *J. Pediat.*, 1936, **6**, 151.

4 Clark, D. E., and Brunschwig, A., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 329.

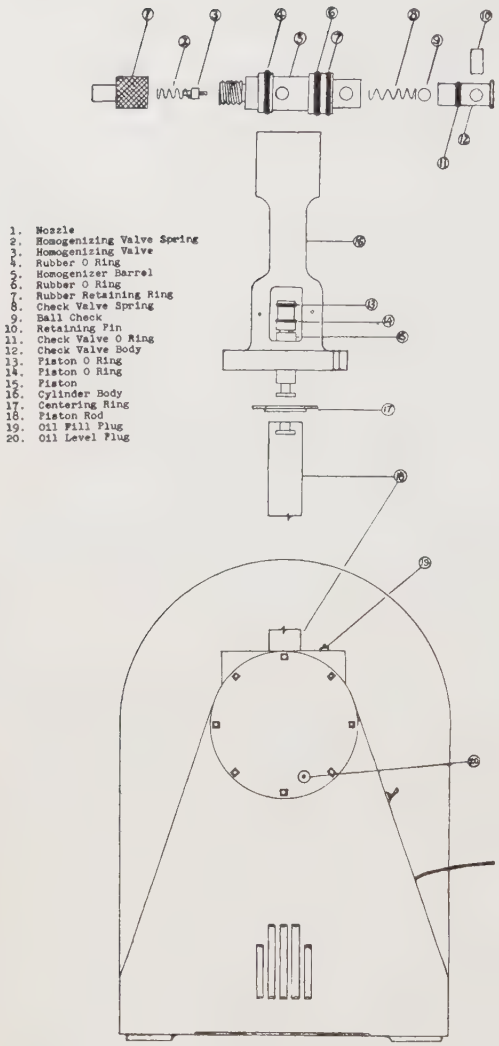

FIG. 1.
Diagrammatic sketch of homogenizer apparatus.

into its cover, a motor and belt mechanism to drive the compressor piston and a homogenizer block fitted with a system of inlet and outlet valves. (See Fig. 1 for diagram of the apparatus.) Within the block a high internal pressure is created which is expended on the homogenization of the mixture. The emulsion is prepared entirely under sterile conditions. The homogenizing block and all the parts in contact with the mixture can be detached as a unit and autoclaved before each batch of homogenate is processed. The ingredients of the mixture already in sterile form are drained from their respective containers into the reservoir tank through a special opening in the cover provided for the purpose. The mixture is converted into a blend or crude emulsion after a 5-minute period of high speed agitation. The blend is then allowed to flow into the homogenizing chamber within which the emulsifying process is completed. The emulsion is continuously recyclized through the block under 2500 pounds pressure for a period of 20 minutes and then collected in sterile liter flasks which are capped and stored ready for intravenous use. Test samples of the homogenate of each batch are examined for particle size and for sterility.

The constituents of the emulsion are solutions of protein hydrolysate (amigen, 10%), 50% glucose, intravenous gelatin (Knox P-20, 6%), and refined coconut oil. All except the latter are available as sterile non-pyrogenic solutions in sealed flasks. The coconut oil is autoclaved before its addition into the tank. The combined fat emulsion used in the present studies is an homogenate of approximately 10% fatty oil, 5% protein hydrolysate, 5% glucose, and 2% gelatin. The caloric value of the emulsion is about 1,300 calories per liter. The pH of the emulsion is about 6.2. The particle size of the emulsion is less than one micron and exhibits active Brownian movement on microscopic examination.

In the present studies intravenous gelatin was used exclusively as the emulsifying agent. After many experiments it was found to be superior to the soy bean phosphatides, lecithin, and a variety of artificial stabilizing agents. Intravenous gelatin *per se* has been proved to be non-toxic and non-allergenic. As an emulsifying agent a relatively small amount, less than 3%, is required for the emulsification of 10% fatty oil. The stability of the 10% combined fat emulsion has been observed for a period of more than two years without "creaming" or "oiling out."

Method of Study. The combined fat emulsion was administered without selectivity to hospital patients who were being treated for a variety of surgical conditions. Many of these patients ran a chronic low grade fever. The studies were started in the morning with

TABLE I.
Immediate Effects from Infusion of One Liter of 10% Combined Fat Emulsion.

Temperature reactions		Constitutional reactions			
Elevation	%	Subjective	%	Objective	%
< 1°	29	Nausea	1	Chills	9
1° to 1.9°	50	Headache	3	Vomiting	2
2° to 2.9°	13	Dizziness	1	Cough	2
> 3°	8	Fatty taste	1	Allergic	1

the patient in the post-absorptive state after a fasting period of 14 hours. Before the infusion was started a sample of blood was taken, the morning urine was obtained, and temperature, pulse, respirations, and blood pressure were noted. The infusion was then given intravenously at a specific rate ranging from 20 to 80 drops per minute varying in different patients. During the course of the infusion and throughout the post-infusion phase until the next morning blood and urine samples were taken and the above mentioned clinical notations were made at regular intervals. The following tests were made routinely on the blood: hemoglobin, red cell count, white cell count with differential, sedimentation time, chylomicron count, and specific gravity. In special cases tests were made for the quantitation of blood lipids with the refractometer and the nephelometer, for blood volume by the Evans blue method and for blood viscosity with the Hess viscosimeter. Clinically, subjective and objective constitutional reactions relative to the intravenous infusion of the emulsion were studied. X-rays of the lungs were taken in series for any lesions due to fatty infiltration, pulmonary irritation or pulmonary emboli.[5] Final check examinations were made 2 weeks after completion of the infusions for hemolytic anemia and for possible liver or kidney damage. Surgical liver biopsies were obtained from several patients who received the emulsion. These specimens were prepared for microscopic analysis by staining with osmic acid, oil-red –O, Sudan IV, and hematoxylin-eosin.

Results. The combined fat emulsion was infused intravenously into 76 human subjects for a total of more than 250 liters. An average of 4 liters of emulsion was administered to each of 35 patients. One patient received a total of 16 liters of the emulsion which was the maximum amount infused into any one patient. Five patients received 2 liters of the emulsion during one infusion period instead of a single liter.

Temperature changes during and immediately after the infusion were computed according to the degree of elevation above the pre-infusion temperature. The results have been classified in Table I on a percentage basis from the protocols of the last 200 infusions. These values were not corrected for temperature changes due to the disease of the patient. Constitutional reactions, subjective and objective, have also been outlined in Table I. The incidence of chills averaged 9% of all infusions, the majority of which came concomitantly with a high temperature reaction. In 2% chills occurred independently, without any temperature elevation. The chill reaction was the severest type of reaction encountered in the present series. Allergenic reactions were usually of the urticarial type due to sensitivity to the amigen component in the emulsion. The latter reaction could be obtained by the infusion of amigen alone and was found to be controllable by antihistaminic drugs given in conjunction with the administration of the emulsion. Cough and vomiting were moderately severe reactions which were usually controlled by reduction of the speed of intravenous administration of the emulsion.

Changes of blood pressure in response to the injection of the emulsion were variable. During the infusion period there was generally a slight elevation of blood pressure followed by a moderate fall in pressure which persisted for 2 hours after completion of the

[5] Jirka, F., and Scuderi, C. S., *Arch. Surg.*, 1936, **33**, 708.

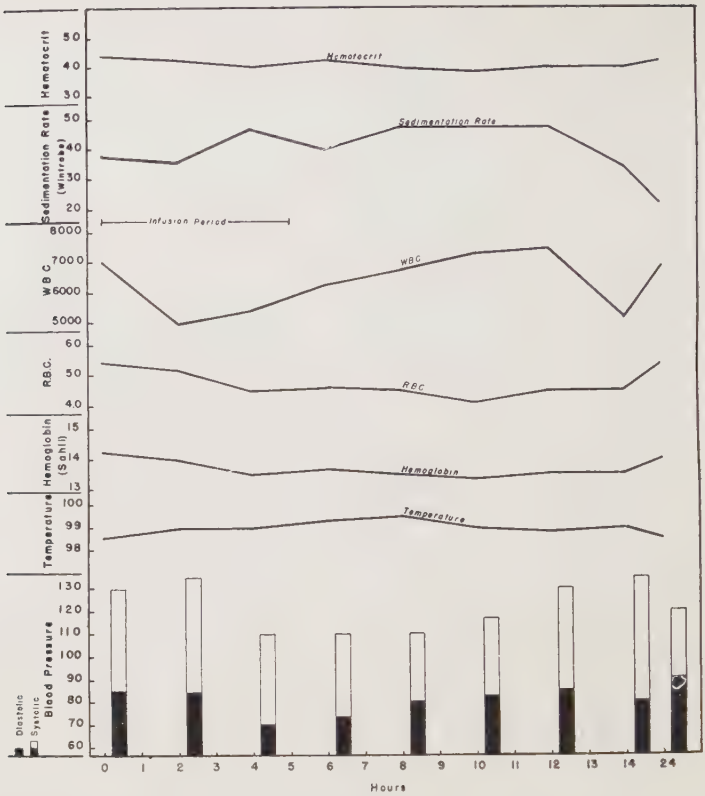

FIG. 2.

Graphs showing the effects of the intravenous infusion of one liter of the 10 per cent combined fat emulsion. The values of the individual curves were averaged from all the protocols.

infusion. The elevation of systolic pressure did not exceed 20 mm Hg and the fall in systolic pressure ranged from 10 to 25 mm Hg. The diastolic pressure tended to fall towards the end of the infusion but also returned to its normal level (Fig. 2).

The values for hemoglobin, hematocrit cell volume and red and white cells tended to fall below their initial values during the infusion phase of the emulsion and returned to their original levels shortly before or after the termination of the infusion. The erythrocyte sedimentation rate showed an increase in rapidity of sedimentation which averaged 35% greater than the pre-infusion sedimentation time, and also returned to its initial level. The differential white count was not indicative of any trend. Leukopenia was found to occur in 2% of infusions concomitant with chill and temperature reactions (Fig. 2 and Table II).

Counts of chylomicrons were made from peripheral venous blood under dark field illumination with a net micrometer in the eyepiece of the microscope. The curve of chylomicron counts beginning from the post-absorptive level through the infusion phase of the emulsion was characteristic. In the post-absorptive state the initial pre-infusion count varied from zero to 10 chylomicrons. After the infusion was started there was a rapid rise in the number of chylomicrons to a peak. At about the mid-point of the infusion the chylomicrons began to decrease in number and return slowly to the initial level. Fig. 3 was drawn from the average values of all the chylomicron counts. The chylomicron curve was found to be modifiable by the speed of infusion, the amount of fat emulsion administered and the oral ingestion of food.

Quantitative microchemical determinations of blood lipids showed a rapid rise of blood fat

TABLE II.
Effects of Intravenous Infusion of One Liter of Combined Fat Emulsion Into a Subject.

Time (min.)	Amt infused (ml)	Temperature Fahrenheit (degrees)	Pulse rate (per min.)	Blood pressure (mm Hg)	Hemoglobin Sahli (g)	Sedimentation time (mm/hr)	R.B.C. count (million)	W.B.C. count	Hematocrit (%)	Plasma refractive index	Chylomicra count ¼ unit	Blood lipids (mg)	Urine acetone
0	—	98.0	80	132/98	14.0	24	4.75	7000	44	1.350	8	800	neg.
60	240	97.8	80										
120	350	98.0	98	140/100	13.0	23	4.75	6800	41	1.349	350	1070	neg.
180	520	98.6	100										
240	625	98.6	96	140/110	13.0	36	4.70	5000	40	1.349	150	905	1+
300	—	98.8	104										
360	1000	98.6	104	140/110	13.2	36	4.75	5600	41	1.351	250	625	1+
420	—	98.2	100										
480	—	99.0	104	130/110	13.4	21	4.90	5800	42	1.350	150	810	neg.
540	—	98.0	104										
600	—	99.0	100	150/110	13.0	36	4.75	6000	41	1.350	100	705	neg.
660	—	99.0	98										
720	—	99.2	100	150/110	13.2	36	4.90	6600	42	1.350	50	770	neg.
24 hr	—	—	—	—	—	28	5.20	6800	43	—	10	685	neg.

and an equally rapid fall to normal limits within 2 hours after completion of the infusion. Nephelometric examinations of plasma samples showed variations in the degree of turbidity comparable to the variations obtained by microchemical analysis and chylomicron counts made on the same samples. Viscosimetric determinations and refractometric analysis showed changes in the blood during the course of the infusion which were consistent with the changes already noted. The specific gravity of the plasma showed a temporary dilution effect due to the infusion followed by a return to normal limits.

Consecutive urine studies were made in 43 infusion tests. In no case was a positive test for blood or urobilinogen obtained which could be attributed to the emulsion. Chemical tests for fat in the urine were negative. Dark field examination of the urine for chylomicrons sometimes showed a moderate elevation in the number of fat particles although the chemical test for fat was negative. A positive reaction was obtained occasionally for acetone varying from 1 + to 2 + in 2 or 3 of the intermediate urine specimens taken during the course of the infusion of the emulsion. When the infusion was completed the positive tests for acetone became negative. A positive test for diacetic acid was found rarely.

Hematologic follow-up studies in patients who received either single or multiple infusions showed no signs of hemolytic anemia. Likewise x-rays of the lungs gave no evidence of fatty emboli or pulmonary irritation. The photographs in Fig. 4 and Fig. 5 were taken to show the microscopic appearance of human liver sections. The first, Fig. 4, was taken a few hours after an infusion and the second, Fig. 5, 36 hours after the last of 9 consecutive infusions of the combined fat emulsion. Liver and kidney function studies showed no pathologic physiology ascribable to the use of the emulsion.

In 5 patients it was necessary to discontinue the infusion of the emulsion before half of the contents of one liter had entered the vein. This was necessary because of the severity of the chill experienced by the patients. All of the 5 patients were in advanced states of malnutrition with severe anemia and with markedly diminished circulating blood volumes. Four of these patients were able to tolerate large volumes of the combined fat emulsion after the anemia and the blood volume were corrected by multiple blood transfusions.

Comments. The combined fat emulsion was administered intravenously to human patients with safety. The immediate and late effects of single and repeated infusions of the

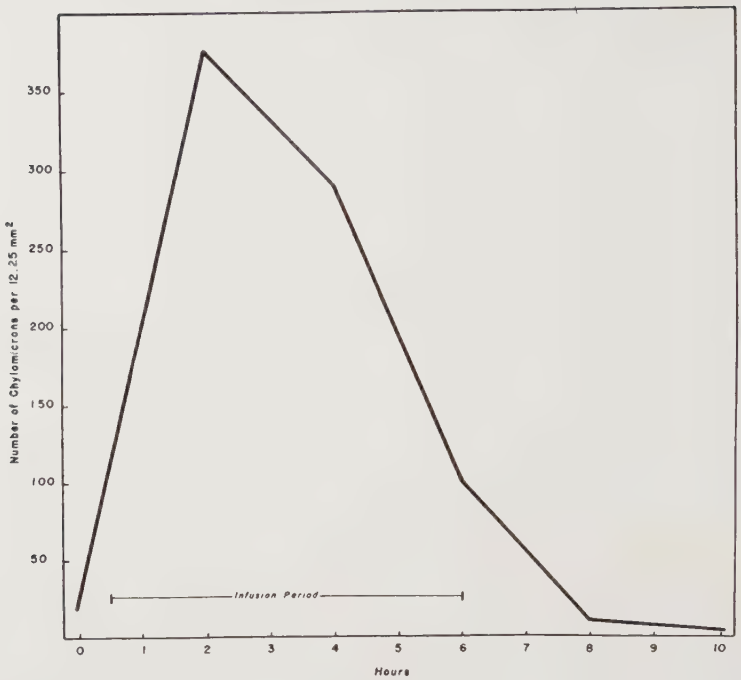

FIG. 3.

Graph of the curve of the chylomicron counts obtained during the infusion and post-infusion periods of the 10 per cent combined fat emulsion. The values on this curve have been averaged from all the chylomicron counts in the protocols.

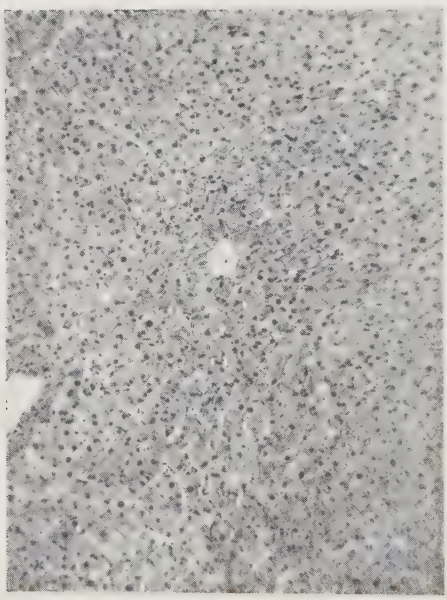

FIG. 4.

Low power magnification of human liver section focused on central vein. Surgical biopsy several hours after patient had received 2 liters of the 10 per cent combined fat emulsion. (H & E stain ×50).

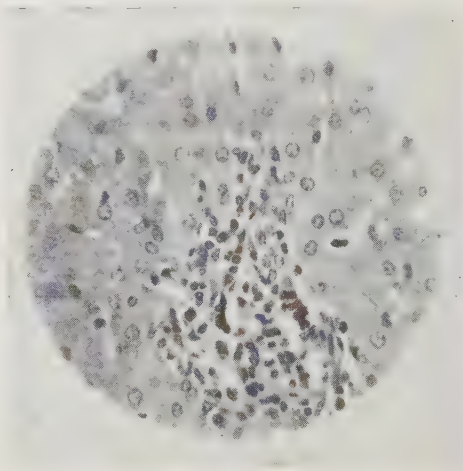

FIG. 5.

High power magnification of human liver section focused on periportal area. (H & E stain ×220). Surgical biopsy 36 hours after patient had received a total of 9 infusions of the combined fat emulsion.

emulsion were studied both from the clinical and the hematologic standpoint. A moderate elevation in temperature as a consequence of

injection of the combined fat emulsion can be accounted for on the basis of an increase in total heat production due to the metabolism of fat plethora. The increased rate of blood sedimentation was believed to be associated with erythrocyte pseudo-agglutination caused by the infused gelatin. The variations in hemoglobin, etc., were related to the dilution effect of the emulsion. The incidence of constitutional reactions compared favorably with reactions encountered after blood transfusions. The sharp rise in the number of chylomicrons followed by the slower fall was indicative of the rate of disappearance of the intravenously injected fat from the blood stream. The appearance of acetone found in an occasional specimen of urine during the infusion phase may be explained on the basis of incomplete oxidation of the fat. In the follow-up we were unable to find any serious toxic effects or late sequelae ascribable to the emulsion.

Summary. The method of preparation of a 10% combined fat emulsion used in the present investigation was described. A combined fat emulsion with a potency of approximately 1300 calories per liter was infused intravenously into 76 human subjects. A variety of laboratory tests and clinical observations provided evidence in favor of its suitability as an intravenous emulsion.

The authors are indebted to the E. F. Drew Co., to the Mead Johnson Co., and to the C. F. Knox Co., for generous supplies of material used for the preparation of the emulsion. The homogenizer was made by the C. W. Logeman Co., New York City.

16923

Influence of Temperature on the Distensibility of the Pubic Ligament.*

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The studies of Hisaw and his collaborators[1,2] have demonstrated that hormonal changes can profoundly influence the distensibility of an apparently physiologically inert tissue such as the pubic membrane within a relatively short time. In the present study we have attempted to determine the relative influence of changes in the physical environment, *i.e.* load and temperature, on the response of the pubic ligament of the guinea pig.

The experiments to be described subsequently, were performed in both intact preparations of non-gravid and in isolated preparations of gravid and non-gravid guinea pigs. In the former series hormonal influences were controlled by ovariectomy and injection of serum from pregnant rabbits. It was soon found that under our experimental conditions such hormonal factors played a lesser part than did slight variations in the environment, as *e.g.* caused by variations in the distance of a source of heat to the preparation.

The method used in studying the effect of temperature on the stretching of the pubic ligament in the intact animal is illustrated in Fig. 1. The animal was kept under deep nembutal narcosis during the entire experiment. Stretch on the pubic ligament was exerted by a weight which pulled a string loop fastened with one end to the pubis, at the other to the ischium. The other half of the pelvis was similarly fastened with a short loop of string to a fixed point. Recording took place with a third loop, fastened to the side on which the weight pulled; this arrange-

* This investigation was carried out with the aid of a grant from the Harlan Shoemaker Fund for Paralytics, Los Angeles, Calif.

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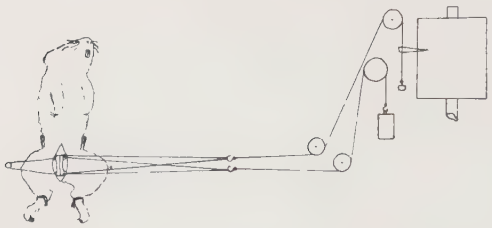

FIG. 1.
Diagram of the arrangement of stretching the pubic ligament of a guinea pig *in vivo*.

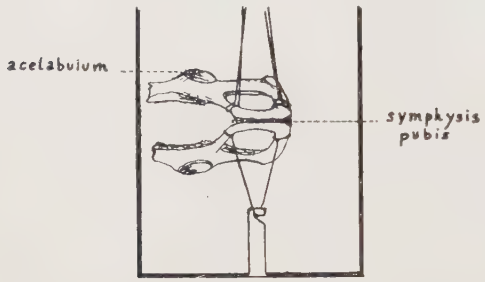

FIG. 2.
Part of the arrangement for the experiments *in vitro*, showing the attachment of the strings to the preparation.

ment made it possible to record more nearly the actual change in the pubic membrane only, without interference of the stretching of the wire by the stretching weight. The animal was held in position between two nails driven just above the hindlegs into the board on which the preparation was mounted and by staples which held the hind legs fastened to the board.

A similar technic was used for the recording of the stretch of the isolated preparation. In this case the pelvis was cut just above the acetabulea. The short single loop on one side was fastened to a hook in the bottom of a cylindrical container, made of brass, which could be submerged in a constant temperature bath. The preparation itself was bathed in mammalian Ringer solution (Fig. 2).

Stretching was recorded on a kymograph and the preparations were subjected to stretch for up to 6 hours in the case of stretching *in situ*, whereas isolated preparations were kept for 12 hours or more. The number of animals in which the ligament was stretched *in vivo* and significant temperature changes were made, was 12. A far larger number, in which the temperature was kept more constant, and in which hormonal factors were varied, served to show that the variations observed with temperature change did not occur without such change. Eight ligaments were stretched *in vitro* under thermostatic conditions.

Results. Table I represents the results obtained when isolated pubic ligaments with the adjacent symphyses, were subjected to a constant load of 100 g at various temperatures for 12 hours. It will be seen that there is no noticeable difference between stretching at 25°C and 30°C. With higher temperatures the rate of stretching increases, and at 38°C is many times that at 25°C. Fig. 3 and 4 represent changes in the rate of stretching within a given preparation when temperature is varied. The stretch at the onset of the experiment is always larger at the same temperature than later, dropping exponentially. By changing temperature this curve can be greatly influenced. Thus from Fig. 3 it is seen that lowering the temperature from 37°C to 34°C almost completely prevents distension from a load of 100 g, but that stretching resumes when the original temperature is reestablished. This experiment was performed on a non-pregnant animal, with the preparation *in situ*. The temperatures measured are those of the Ringer fluid which surrounds the membrane. This fluid also prevents drying of the membrane. The experiment shown is representative of many performed in this way. From these it follows that a greatly increased rate of stretching is caused by a temperature rise between 33 and 36°C. Below this range in the non-pregnant guinea pig there is very little stretching with a load of 100 g, whereas above this range (up to 42°C) the rate further increases, but not so much.

Fig. 4 represents the stretching *in vitro* of

TABLE I.
Change in Distances of Isolated Pubic Membranes After 12 Hours of Stretching by 100 g virgin guinea pigs.

Temp.°C	Stretch in mm*	mm per hr
25	20	1.67
30	20	1.67
35	68	5.08
38	138	11.5

* As recorded on drum; about 4 times actual.

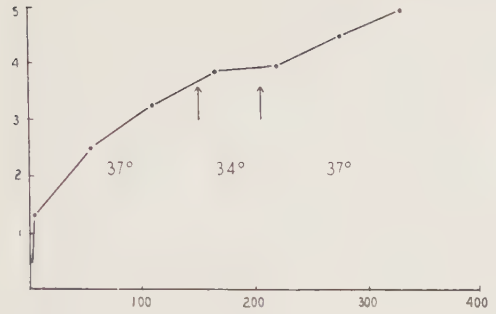

FIG. 3.

Plot of the increase in distance of the membrane, subjected to a stretch of 100 g *in vivo*, of a virgin guinea pig, weight 500 g. Between arrows temperature lowered from 37 to 34 degrees. Length in arbitrary units, each of which corresponds to about ½ mm. Time in units of 100 min.

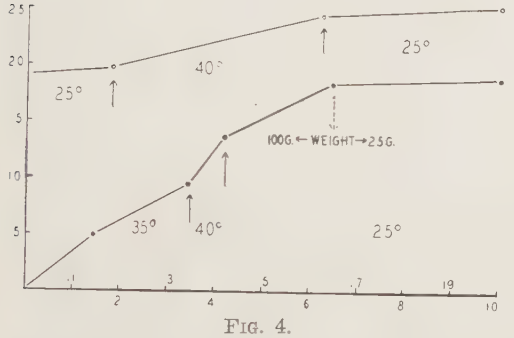

FIG. 4.

Stretching curve obtained from ligament of a pregnant animal *in vitro*, showing the influence of temperature and weight. Notice the much greater stretch than in Fig. 3. Upper curve is continuation of lower curve. Time in hr.

a ligament from an animal almost at term, to which 100 g stretching weight was applied. There is an increase in rate of stretch by changing from 35 to 40°C. In this pregnant animal lowering to 25°C does not prevent a very noticeable stretching. Even decreasing the stretching load to 25 g only reduces the stretching at 25°C to a value comparable to that found in non-pregnant pigs at body temperature. At 40°C the amount of stretching with 25 g load is again considerably increased.

This much greater sensitivity of the membrane of the pregnant animal is completely in accord with the view that the pressures present in the pelvis will be sufficient to push the bones apart. It is difficult to judge, how much pressure might occur under non-pregnant as compared to under pregnant conditions, the much smaller distensibility obviously prevents in most animals any great separation of the bones. Another factor which must be considered in these experiments is the presence of ligaments joining the pelvic girdle to the spinal column. These do possibly participate in the experiments on the membrane *in situ*, but not in the case of the isolated preparations. This may be the main reason, why the stretching curves of the latter were always considerably freer from small irregularities than the former.

It may be pointed out that the great influence of temperature changes between 33 and 37°C on the stretching of the pubic ligament raises interesting questions concerning the behavior of other ligaments in the body, which, by their location might be subject to temperature changes in this range under normal living conditions.

Summary. The effect of temperature on the distensibility of the pubic ligament of the guinea pig was investigated in intact and isolated preparations using a constant load. Both types of preparations exhibited a marked dependence of stretching on temperature between 30 to 40°C with the mode lying in the range 34-35°C under conditions of moderate loading.

Distension at 30°C is negligible, while at temperatures above 34 it is rapid. Preparations from pregnant animals show a lower threshold both for temperature and weight.

The authors want to thank Dr. D. R. Howton and Mr. H. H. Crawford for their assistance during the earlier part of this investigation.

16924

Degranulation of Beta Cells of Rat's Pancreas by Glucose Correlated with Alterations in Glucose Tolerance.*

Carl A. Peterson. (Introduced by B. J. Clawson.)

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After the relationship of the pancreas to diabetes had been established by Von Mering and Minkowski,[1] and before the discovery of insulin, Allen[2] and Homans,[3] working independently, demonstrated changes in the beta cells of the islands of Langerhans in partially depancreatized diabetic dogs. Allen postulated that these observed alterations would be reversible, and Copp and Barclay,[4] after the discovery of insulin, proved this hypothesis to be correct by observing the recovery of the beta cells in partially depancreatized diabetic dogs treated with insulin. Allen explained this phenomenon by postulating that the beta cells were the source of the antidiabetic hormone and that an excessive functional demand caused an exhaustion of these cells and produced the histological changes he observed—degranulation and vacuolization. These early observations, verified and amplified by later research, were made using the Lane Bensley technic for staining the beta cells. If one can judge from the photomicrographs included in these early reports this method gave very inadequate results and the staining would not compare in quality or accuracy with the results produced by the Gomori stain[5] which was used exclusively in these experiments.

More recently diabetes has been produced experimentally by methods other than pancreatectomy; by the injection of the hormones of the anterior pituitary[6] and thyroid,[7] by the injection of alloxan,[8] and by the injection of large amounts of glucose.[9] And when a diabetic state has been established alterations in the beta cells of the pancreas, degranulation, vacuolization, and necrosis, are invariably found to be present.

In other experimental methods the changes are limited to degranulation of the beta cells and diabetes does not develop. Starving a white rat or feeding it an exclusively fat diet will completely degranulate the beta cells of the pancreas.[10] Repeated daily injections of protamine zinc insulin in the rat will produce degranulation of the beta cells.[11,12] Gomori[13] and associates demonstrated variable degranulation of the beta cells in the guinea pig following a single intraperitoneal injection of glucose.

The present investigation is concerned with the changes produced in the beta cells in the pancreas of the white rat and the alterations in the glucose tolerance following a single intra-cardiac injection of glucose.

Materials and methods. White rats of the Sprague-Dawley strain were used exclusively. Rats of various ages and weights and of both sexes were used. Glucose was given in 20%

* This work has been done under a grant from the Office of Naval Research made to Dr. E. T. Bell and a Research Fellowship from the U. S. Public Health Service to Dr. Carl Peterson.

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TABLE I.
Results of One Injection of Glucose into Blood Stream of White Rat.

Rat No.	Sex	Wt, g	G glucose/kg	Hr inj. to autop.	Beta gran.	Rat No.	Sex	Wt, g	G glucose/kg	Time inj. to autop.	Beta gran.
20	M	200	0.25	1	3	307	M	300	3	5 min.	3
X	M	230	0.5	2	3	308	M	340	3	10 min.	1
E	F	130	1	¼	3	R	M	280	3	15 min.	0
D	F	160	1	½	3	Q	M	420	3	30 min.	0
C	M	120	1	¾	3	P	F	310	3	45 min.	0
B	F	185	1	1	2	303	M	330	3	12 hr	0
A	M	150	1	1½	2	310	F	300	3	24 hr	0
K	M	220	2	1	1—	T	M	280	3	24 hr	1
L	F	190	3	1	0	U	M	400	3	48 hr	2
M	M	250	4	1	0	311	F	320	3	48 hr	3
N	M	210	6	1	0	V	M	400	3	72 hr	3
O*	M	360	3	1	0	Z*	F	230	3	1 hr	0

* 3 units of crystalline zinc insulin per 1.0 g glucose added to syringe.

solution in distilled water via needle and syringe directly into the left ventricle of the heart of the rat previously anesthetized with ether. Blood for glucose determinations was withdrawn by the same method and the Folin-Wu micro-method was used for the determinations. The rats were sacrificed at various intervals by injecting air into the left ventricle of the heart. All pancreases were fixed in Bouin's fixative and stained with Gomori's stain. A pancreas from a normal rat was included with each staining and all estimations of partial degranulation were made with reference to this control tissue. The degree of granulation was graded from 3, representing a pancreas with all the beta cells fully granulated, to 0, a totally degranulated pancreas. The designation 1 indicates a pancreas with very marked degranulation of the beta cells while 2 indicates a definite partial degranulation but not as marked as 1. The glucose tolerances were determined by injecting 0.25 g per kilo body weight of 20% glucose into the left ventricle of the heart.

Several experiments were attempted: Glucose in concentrations from 0.25 g per kilo body weight to 6.0 g per kilo was injected. Rats were then sacrificed at intervals from 5 minutes to several days. Glucose tolerances, blood glucose levels, and urine sugars were determined at varying intervals. All determinations of glycemia were made in the fasting state; all urine sugar recordings were determined on overnight specimens. In 2 rats 3 units of crystalline zinc insulin per 1.0 g of glucose were added to the syringe and injected simultaneously with the glucose. One rat was given 12 single injections of glucose, 3.0 g per kilo, over a period of 2 weeks.

Results. The beta cells of the islands of Langerhans were invariably totally degranulated within 15 minutes after the injection if 3.0 g per kilo, or higher concentrations of glucose were used. Lesser concentrations gave variable results: Less than 1.0 g per kilo resulted in no perceptible degranulation; 1.0 g per kilo reduced the granulation to the level of 2 in one hour; 2.0 g per kilo reduced the granulation to 1 or 1— at the end of one hour.

This process of degranulation was found to be reversible; regranulation of the beta cells followed degranulation. However, a delay of about 48 hours was observed. Twenty-four hours after the injection the granulation was found to remain markedly reduced (0-1); but in 48 hours the granulation appeared to be almost complete (2-3).

Glucose tolerance studies on rats 24 hours after the single injection of glucose (3.0 g per kilo) revealed curves of the diabetic or potential diabetic type (Fig. 1a). One week after the injection the glucose tolerance had returned to normal.

When 3 units of crystalline zinc insulin per 1.0 g of glucose were added to the syringe a marked reduction in the hyperglycemia followed, but not for approximately 30 minutes during which interval degranulation of the beta cells took place (Fig. 1b).

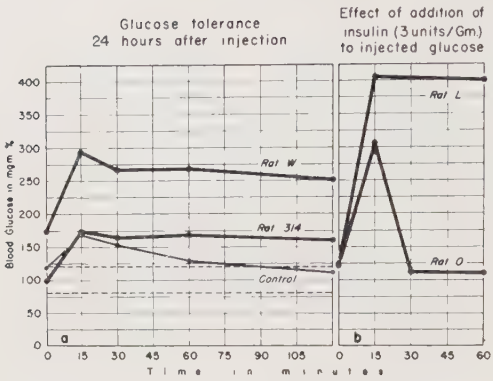

FIG. 1a.

Rats W and 314 received injection of glucose, 3.0 g/kg, 24 hr previous. The curves represent upper and lower extremes of glucose tolerance. Most other determinations fell in between.

FIG. 1b.

Rats L and O were both given glucose 3.0 g/kg. Zinc crystalline insulin, 3 units per g of glucose, was added to the syringe in injecting rat O.

Fasting blood sugars, determined 24 hours after the injection, were found to be elevated in almost every instance. One rat, No. 314, which showed a fasting blood sugar within the normal range, was found to have a glucose tolerance curve of a potential diabetic type (Fig. 1a). As would be expected most rats also showed a marked glycosuria 24 hours after the injection. This usually disappeared by 48 hours.

From these observations it became apparent that a correlation existed between the degree of beta cell granulation, the level of glycemia, the type of glucose tolerance curve, and the degree of glycosuria.

Attention is again directed to rat 314, glucose tolerance of which is illustrated in Fig. 1a. Following this determination, which as previously noted consists of the injection of 0.25 g of glucose per kilo, this rat showed a daily glycosuria of 2+ for one week. The possible significance of this observation is discussed below.

The one rat, No. Y, which was given 12 daily single injections of glucose, 3.0 g per kilo, showed a persistent elevation of fasting blood sugars and marked glycosuria for 8 days after cessation of the injections—a temporary diabetic state.

It should be noted that all control animals, treated exactly as the above animals with the exception that no glucose was injected, showed no degranulation, hyperglycemia, glycosuria, or abnormal glucose tolerances.

Discussion. The injection of a large amount of glucose into the blood stream of the rat, as described above, undoubtedly causes an excessive functional demand upon the beta cells of the pancreas leading to complete exhaustion of the cells—the degranulated state. The stimulus initiating this exhaustion of the cell is of necessity the injected glucose. And from the data recorded in these experiments and from the work of Gomori and associates,[13] it is evident that the greater the concentration of the stimulating agent in the blood the greater the amount of beta cell secretion.

The beta cell in its completely exhausted state is not organically injured as determined by histologic studies—it shows no alterations other than the degranulation. However, physiologic studies as reported herein would indicate that the cell is functionally impaired. About 48 hours are required for the recovery of the cell as measured by glucose tolerance, levels of glycemia, and degree of glycosuria. Repeated injections, as exemplified by rat Y, apparently cause an accumulative functional impairment upon the beta cells; the beta cells recover within 48 hours after one injection but require one week to recover after 12 injections. Theoretically, continued injections might transform the temporary functional impairment into a permanent deficiency resulting in diabetes. This feature is being investigated.

The sensitivity of the beta cells to overstimulation probably varies somewhat from one animal to another. Rat 314 is an example of marked sensitivity to a slight stimulus following the production of functionally impaired beta cells. The day following the intra-cardiac injection of 3.0 g glucose per kilo, rat 314 had a fasting blood sugar within normal range but showed a glucose tolerance curve of a potential diabetic type, Fig. 2a. In doing the glucose tolerance 0.25 g of glucose per kilo was again injected. For one week following this procedure the rat showed a marked daily glycosuria and an elevated fasting blood sugar. Thus a small extra demand upon a previously functionally impaired

TABLE II.
Correlation of Beta Granulation with Blood Sugar, Glucose Tolerance, and Glycosuria Following One Injection of Glucose (3.0 g/kg).

Hr after injection	0	1	24	48	72	Week
Beta granulation	3	0	0-1	2-3	3	3
Avg blood glucose	118	390	163	122	108	106
Glucose tol. curve	Normal		Diabetic or Pot. diab.			Normal
Glycosuria	0	4+	2+ to 4+	0 to Tr.	0*	0*

* One exception, Rat 314, 2+ daily glycosuria for one week.

beta cell produced the picture of a temporary diabetic state.

The fact that the beta cells eventually regranulate completely is also strong evidence that the functional impairment is only temporary. The final proof, of course, for the theory of functional impairment due to overstimulation is the work of Lukens and Dohan,[9] in which cats were made permanently diabetic by the administration of large amounts of glucose.

The marked correlation between the degree of beta cell granulation with alterations of glucose tolerance, elevation of the fasting blood sugars, and the presence of glycosuria would confirm the widely held opinion that the granules are a form of insulin. The phenomenon of complete degranulation within 15 minutes in response to the stimulus of injected glucose would be further evidence for this opinion.

The addition of crystalline zinc insulin to the injected glucose resulted in an interesting observation. We had anticipated that the exogenous insulin would protect the beta cells from degranulation but interval blood sugars explained the results. The beta cells were apparently degranulated during the initial period of hyperglycemia, as shown in Fig. 1b. An initial lag in the effect of the injected insulin is well demonstrated by the blood sugar curves. The significance of this phenomenon has not yet been determined.

Summary. The beta cells of the rat's pancreas can be completely degranulated within 15 minutes by a single intra-cardiac injection of glucose. Dosage: 3.0 g glucose per kilo body weight.

The phenomenon of degranulation is reversible; regranulation follows degranulation, but requires about 48 hours.

Rats with degranulated beta cells show a reduced glucose tolerance, elevated fasting blood sugars, and glycosuria.

The addition of 3 units of crystalline zinc insulin per 1.0 g of glucose does not prevent the degranulation of the beta cells due to a delay in the effect of the injected insulin and the consequent production of a hyperglycemia.

Repeated injections of glucose cause a functional impairment of the beta cells as evidenced by the production of a temporary diabetic state.

16925

Effect of Epinephrine in Decreasing Number of Circulating Mononuclear Leucocytes in the Rat.*

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Recent evidence suggests that the number of circulating mononuclear leucocytes is under the control of adrenal cortical hormones[1,2] since these cells are decreased when an excess of cortical hormone is present and increased after adrenalectomy. Epinephrine has been reported to stimulate adrenal cortical secretion.[3-5] Epinephrine administration should, therefore, cause a decrease in the mononuclear count of the blood. Lymphopenia following epinephrine administration has recently been reported to occur in intact but not adrenalectomized dogs.[6]

The problem remains whether epinephrine changes the blood picture by direct action on the adrenal cortex or whether the mechanism is mediated through the pituitary gland. In an effort to add evidence to this point, the effect of epinephrine on the mononuclears of rat blood was determined in the presence or absence of the endocrine glands in question (pituitary and adrenal).

The results indicate that epinephrine administration is followed by a decrease in the number of circulating mononuclear leucocytes in normal, adreno-demedullated, and hypophysectomized rats but not in adrenalectomized rats.

Methods. Healthy growing male rats of the Long-Evans strain, 60 to 70 days of age and kept under similar environmental conditions were employed. They received the regular stock diet (XIV)† *ad libitum* supplemented twice weekly with fresh lettuce.

Hypophysectomy was performed under ether anesthesia. Adreno-demedullation and adrenalectomy were performed under sodium pentobarbital anesthesia. Hypophysectomized and adrenalectomized rats were used on the tenth postoperative day. Adreno-demedullated rats were used 20 or 30 days after the operation. Adrenalectomized rats were maintained on drinking water containing 1% sodium chloride.

Blood counts were made from tail vein blood. The smears were stained with Wright's stain and between 100 and 200 leucocytes were counted. White blood cells were classified as mononuclear or neutrophilic leucocytes.

Epinephrine chloride, 1:1000 (Parke Davis and Co.), was injected subcutaneously in the amount of 0.07 cc per 100 g body weight. For the purpose of control, saline solution (0.9%) was injected subcutaneously in the amount of 0.07 cc per 100 g body weight.

Results. Fifteen to 30 minutes after administering epinephrine to normal rats (Table I) a slight but consistent elevation in the number of mononuclear leucocytes occurred. After one hour, the number of mononuclears began to decrease, reaching the lowest level in 2 hours. Thereafter, the number of mononuclears began to increase, reaching the normal level after 6 to 8 hours. Neutrophilic leucocytes increased rapidly throughout the

* Aided by grants from the Board of Research of the University of California, Berkeley, and the Rockefeller Foundation, New York City.

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† Diet XIV (stock diet) consisted of ground whole wheat 67.5%, casein 15.0%, whole milk powder 10.0%, NaCl 0.75%, $CaCO_3$ 1.5%, hydrogenated vegetable oil (Crisco or primex) 5.25%. To each kg of diet were added 3.5 g of sardilene (fish oil concentrate containing 3000 USP units of vitamin A and 400 chick units of vitamin D per g).

TABLE I. Number of Circulating Leucocytes in the Rat After Administration of Epinephrine or Saline Solution.

Treatment	Experimental animals				Mononuclear counts — Hr after injection				Neutrophil counts — Hr after injection			
	Type	No.	B.W. (g)	Age (days)	0	½	2	4	0	½	2	4
Epinephrine chloride	Normal	10	233	71	11600 (1300)	11800 (1150)	5500* (750)	9000 (1600)	2900 (500)	3750 (1050)	7850* (1200)	15200* (1650)
	Adrenodemedullated	8	245	68	10400 (550)	9400 (1100)	6200* (300)	9400 (900)	2700 (350)	3300 (450)	8600* (850)	16000* (1900)
	Adrenaleetomized	10	247	66	13600 (2100)	17600 (2000)	15400 (1350)	19400 (2200)	4250 (850)	4800 (550)	11300* (1250)	17100* (2200)
	Hypophysectomized	8	205	70	22000 (1400)	19200 (750)	14000* (1400)	18000 (2100)	3000 (350)	4000 (700)	5000† (650)	10900* (1250)
0.9% NaCl	Normal	10	262	69	10750 (900)	7550† (650)	7300* (600)	10650 (900)	2600 (400)	2600 (450)	3900† (400)	5250† (850)
	Adrenodemedullated	8	218	60	10150 (600)	9300 (750)	12000 (1000)	13000† (1100)	2700 (300)	3850† (300)	5050* (350)	5500* (500)
	Hypophysectomized	8	180	65	15200 (950)	15300 (800)	17700 (1000)	23000* (1400)	3200 (400)	4000 (450)	5200 (800)	9600* (950)

Leucocyte counts are expressed as the number per mm^3 (mean value with standard error in parenthesis).
* Denotes highly significant values ($p = <.01$) when compared with the 0 hour count for that group.
† Denotes probably significant values ($p = <.05$) when compared with 0 hour count for that group. Other values presented are not significant when compared in this way.

entire period. Twenty-four hours later the number of both types of leucocytes was slightly lower than the pretreatment number. Essentially the same response was obtained by injecting epinephrine into adreno-demedullated rats. Having established that the minimum level in the number of circulating mononuclear leucocytes was reached 2 hours after administering epinephrine, blood counts were restricted to a 4-hour period and were made at the intervals shown in the table.

Two hours after administering epinephrine to hypophysectomized rats, the number of mononuclear leucocytes was significantly decreased, although the percentage decrease was not as great as in the epinephrine treated normal rats. The number of neutrophils increased throughout the 4-hour period.

In adrenalectomized rats the number of mononuclear leucocytes did not fall but was slightly increased after administering epinephrine. The number of neutrophils increased as in the other groups.

In order to determine the error which might be incurred by repeated blood counts taken from the tail, and from handling the rats, 0.9% saline solution was injected into normal, adreno-demedullated, and hypophysectomized rats and blood counts were taken at the same intervals. Although the number of mononuclears was decreased in normal rats injected with saline solution, this change was not of the same magnitude as that following epinephrine injection. (It probably can be attributed to stimulation of the adrenal cortex by discharge of epinephrine resulting from excitement.) This decreased count was not observed in adreno-demedullated or hypophysectomized rats after injection of saline solution.

Statistical evaluation. The pretreatment value (0 hour) was considered as normal for the group in question and subsequent values for that group were compared with this initial count in calculating P values.[7] A change in the leucocyte count was considered highly significant if the P value was 0.01 or less and

[7] Fisher, R. A., and Yates, F., *Statistical Tables,* Oliver and Boyd, London, 1938.

probably significant if P was 0.05 or less (Table I).

It was not surprising that the initial mononuclear counts in different groups were not the same as it is well known that mononuclear leucocytes are increased after adrenalectomy or hypophysectomy. Comparison between groups, therefore, was not possible without converting all values to a percentage figure of the pretreatment count which would thus give an initial common basis for comparison. Percentage figures are not presented since this procedure did not alter the conclusions or significance of the data.

Discussion. Harvey[8] reported an increase in the mononuclear count 15 minutes after injection of epinephrine into rabbits. Bjure and Svensson[9] reported an increase after 30 minutes but a decrease after 2 hours in normal human beings. Fegler[10] reported a leucopenic phase 2 hours following administration of epinephrine to dogs. Martin[11] reported a lymphocytosis in human patients but did not make blood determinations longer than 40 minutes after injection of epinephrine. Camp[12] reported a lymphopenia but did not specify the time interval elapsing after injecting epinephrine. Harlow and Selye[13] reported a relative lymphopenia after administering epinephrine twice daily for 2 days. The effect of epinephrine in elevating the blood sugar may be related to the changes in the mononuclear counts. Following glucose administration, Elmadjian, Freeman, and Pincus[14] reported a lymphopenia which was lowest when the blood sugar was highest. This response could not be obtained in adrenalectomized rats.

Two hours after intravenous injections of epinephrine into intact dogs, Malmejac, Chardon, and Gros[6] reported a lymphopenia. The number of lymphocytes returned to the normal level after 4 to 6 hours. A slight lymphocytosis developed after injecting epinephrine into adrenalectomized dogs. These are the same time relationships which are reported in this paper following subcutaneous injection of epinephrine into rats.

The dose of epinephrine employed in the experiments reported here is admittedly too high to be within physiological limits. It is to be pointed out that epinephrine gave the responses indicated only in the presence of the adrenal gland. No attempt was made to determine the minimum effective dose to produce these effects.

Conclusions. The administration of epinephrine to normal or adreno-demedullated rats produced within 2 hours a definite fall in the number of circulating mononuclear leucocytes. The number of mononuclears did not decrease after administering epinephrine to adrenalectomized rats. This indicates that the presence of the adrenal cortex is necessary for epinephrine to cause these effects. After administering epinephrine to hypophysectomized rats, the number of mononuclears decreased as in the epinephrine treated normal rats although the percentage decrease was less. This would seem to indicate that at least one effect of epinephrine is to act directly on the adrenal cortex, releasing substances which lower the level of circulating mononuclear leucocytes.

Since this paper was submitted for publication, Gellhorn and Frank[15] have reported that epinephrine causes a reduction in the number of circulating lymphocytes in normal but not in adrenalectomized rats.

This work is the result of experiments initiated under the direction of Dr. William O. Reinhardt. Acknowledgment is made to Dr. Herbert M. Evans for advice and suggestions.

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15 Gellhorn, E., and Frank, S., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 426.

16926 P

Activation of Proplasmin by a Tissue Fraction.

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The proteolytic enzyme plasmin (fibrinolysin) is found in normal blood plasma in an inactive form, called proplasmin. Activation can be produced by treatment with chloroform, ether and some other organic solvents,[1] as well as by streptokinase.[2] The active form of the enzyme is occasionally found in the blood of certain types of patients,[3] but the mechanism of activation in these cases and whether there exists a physiological mechanism of activation of the enzyme in the normal organism, are at present unknown.

The evidence presented in this report indicates that an activator is present in tissue extracts which could constitute the normally occurring activator of the enzyme.

The lungs of the freshly killed adult rats were removed and forced through a 1 mm mesh. The tissue pulp was ground in a mortar, suspended in 30% sucrose solution[4] and then submitted to differential centrifugation in the cold. Tissue fractions and supernatants were obtained and labeled according to the nomenclature of Claude.[5] For testing the material, the substrate used was 0.2% fibrinogen solution in 0.01 M phosphate buffer in saline at pH 7.2, kept frozen at –10°C, prepared by the method of Ware *et al.*[6] The tissue fraction was mixed with either human blood serum or a globulin solution prepared by precipitating human blood plasma at 45% saturation of ammonium sulfate at pH 7, dissolving the precipitate in a volume of saline equal to ¼ the volume of plasma, and dialyzing against saline for 3 days. The serum or the globulin solution served as a source of proplasmin. Fibrinogen was then added and the mixture was clotted by the addition of thrombin (Lederle clotting globulin) on a glass rod. Time of dissolution of the clot at 37°C was noted.

Table I shows the activity of the different fractions from lung tissue. The presence of inhibitory action was evident in tubes 2 and 6, but no inhibitory action was noted on increasing the amount of microsome fraction (tubes 7 and 8). Control experiments showed that the tissue fractions without addition of globulin solution or the globulin solution alone had no dissolving action on the fibrin clot within the time of observation (2 hours) but occasionally the tissue fractions without addition of globulin exhibited such activity over a period of 16 to 24 hours. This may have been due to slight contamination of the fibrinogen preparation with proplasmin.

Fresh blood serum could be used instead of the globulin solution as a source of proplasmin with essentially similar results.

Conclusion and summary. The data indicate that on mixing tissue pulp from the lungs of freshly killed adult rats with blood serum or a fraction of blood serum containing proplasmin, fibrinolytic activity appeared in the mixture. This was presumably due to activation of proplasmin of serum by a tissue kinase present in the lung tissue. The kinase activity was found to be concentrated in the microsome fraction of the tissue, while the inhibitory activity was in the supernate. The

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TABLE I.
Action of Tissue Fractions on Proplasmin.†

	Reagents in cc.*					
Tube No.	Total extr.	Mitochondria	Supernate 1	Microsomes	Supernate 2	Diss. of clot (min.)
1	.1					15
2	.3					27
3		.1				30
4		.3				35
5			.1			16
6			.3			26
7				.1		11
8				.3		9
9					.1	120+
10					.3	120+

† All fractions were suspended in a volume equal to that of the original extract.
* Fibrinogen 0.2 cc + globulin solution 0.1 cc + 0.01M phosphate buffer 0.3 cc, thrombin (on glass rod) added to each tube. Temp. 37°C; pH 7.2.
Control experiments: 1. Same as in table with omission of globulin solution: no dissolution in 2 hr. 2. Same as in table with omission of fractions of tissue: no dissolution in 2 hr.

system described here may be identical with that proposed by Astrup and Permin.[7] It is known also that the microsome fraction of lung tissue contains most of the thromboplastic activity of the total tissue extract,[5] although this should not be construed as indicating an identity between the kinase and thromboplastin.

[7] Astrup, T., and Permin, P. M., *Nature*, 1947, **159**, 681.

16927

Exoerythrocytic Stages of *Plasmodium cynomolgi* in the *Macaca mulatta*.*

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The exoerythrocytic cycle from sporozoite to erythrocytic parasite has now been demonstrated in 4 species of avian malaria.[1-5] The exoerythrocytic stages, called cryptozoites and metacryptozoites by Huff and Coulston,[4] are widely distributed throughout the tissues of the avian host and develop in cells of the lymphoid-macrophage system and in endothelium.

Recently, Shortt and Garnham,[6,7] and Hawking[8] described pre-erythrocytic stages in the livers of monkeys (*Macaca mulatta*) in-

* This study was supported in part by a grant-in-aid from the United States Public Health Service.

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[6] Shortt, H. E., and Garnham, P. C. C., *Nature*, London, 1948, **161**, 126.

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fected with sporozoites of *P. cynomolgi*. Later Shortt, Garnham, Covell, and Shute[9] found pre-erythrocytic forms of *P. vivax* in a liver biopsy of a man on the seventh day following infection with sporozoites. In the case of *P. cynomolgi* it was suggested that the exoerythrocytic stages developed in hepatic cells, and it was reported that these parasites were not observed in any other organ or tissue of the monkey. Although Hawking, Perry, and Thurston[10] described a pre-erythrocytic parasite in the brain of a monkey inoculated intracerebrally with sporozoites, they considered this parasite to be growing in an abnormal location. Further evidence was presented by Huff and Coulston[11] indicating that the pre-erythrocytic stages of mammalian malaria may develop in cells other than hepatic cells. They inoculated sporozoites of *P. vivax* into the liver of *M. mulatta* and observed cryptozoites in cells of the liver. These cells could not be definitely identified in their preparations but appeared to be either true endothelial cells or possibly cells of the lymphoid-macrophage system.

Materials and methods. Studies on the exoerythrocytic cycle of *P. cynomolgi* in *M. mulatta* have recently been initiated in this laboratory. The strain of *P. cynomolgi* used was received from Dr. R. J. Porter, School of Public Health, University of Michigan, in June 1946. Sporozoites were obtained from *Anopheles quadrimaculatus* which had been fed 15-19 days previously upon infected monkeys.

In these experiments a total of 12 monkeys have been used; 9 were infected with sporozoites, and 3 were controls. Pertinent data on these animals are presented in Table I. In some cases mosquito salivary glands, heavily infected with sporozoites, were inoculated directly into the liver.[4] Afterwards, this marked area was biopsied and serially sectioned. Sporozoites for intravenous inoculations were obtained by grinding in a glass mill either the thoraces of mosquitoes with intact salivary glands or the dissected salivary glands. A series of control experiments was done in which normal mosquito tissue was introduced into monkeys in the same manner as the infected material mentioned above.

The monkey tissues were fixed in Zenker-formalin, embedded in paraffin, and stained by either the Maximow method[4] or the modified Giemsa method described by Shortt and Cooper.[12]

Results. As a result of these investigations, pre-erythrocytic stages of *P. cynomolgi* have been found in the liver and spleen of *M. mulatta*. The experimental data are presented in Table I.

In *Exp. 1,* a monkey was inoculated intravenously with the ground thoraces of 178 heavily infected mosquitoes and was sacrificed and necropsied 6 days and 20 hours later. Upon histological examination of the tissues, several small segmenters and schizonts were observed in the liver in Kupffer cells (Fig. 1) and in the spleen in large mononuclear phagocytic cells, probably reticular in nature. No parasites were found in cells which could definitely be called hepatic cells, but this should not rule out the possibility that such cells may have been infected.

In *Exp. 2,* a monkey was inoculated twice directly into the liver with 18 and 24 heavily infected salivary glands. Biopsies of these inoculation sites, at 47 hours and at 89 hours, disclosed a few schizonts (12-18 μ) some of which appeared to be in Kupffer cells. These pre-erythrocytic stages were always found near the site of inoculation.

Nine days and 18 hours after inoculation, the day following detectable parasitemia, another liver biopsy was taken above the 47-hour inoculation site. In this specimen numerous parasites were found in the liver sinusoids in a circumscribed area. These stages were either schizonts and segmenters or merozoites free in the lumen of the sinusoid and were often associated with the Kupffer and littoral cells. Other parasites

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TABLE I.

Biopsies and Necropsies of Normal Monkeys and Monkeys Infected with Sporozoites of *P. cynomolgi*.

Exper. No.*	Mode of sporozoite inoculation	No. of infected or uninfected mosquitoes used	Site of biopsy	Time of tissue collection: Between inoculation and biopsy (days)	Between inoculation and biopsy (hr)	Between inoculation and necropsy† (days)	Between inoculation and necropsy† (hr)	Pre-erythrocytic stages present	Remarks‡
				Infected Monkeys.					
1 A	Intravenous	178				6	20	Yes	Schizonts and few segmenters. Some in spleen.
2 A	Liver	24	Liver	1	23			Yes	Few schizonts. Parasitemia on 8th day.
B	,,	18	,,	3	17			Yes	Few schizonts.
C			,,	9	18			Yes	Segmenters; biopsy taken near 2 A.
D			,,	9	18			No	Biopsy of normal lobe.
E			Spleen	11	18			?	Large bodies resembling segmenters.
3 A	,,	28	Liver	3	22			Yes	Schizonts and a few segmenters. Parasitemia on 9th day.
B	,,	27	,,	6				No	Injection site not located.
C						14		—	Unstudied.
4 A			,,	1	20			Yes	Small trophozoites.
B	Intrav.	223				5	19	Yes	Schizonts in liver smears.
				Uninfected Monkeys.					
5 A			,,					No	Biopsy of normal monkey.
B		0	Spleen					No	
6 A	Liver	20	Liver	2	3			No	Control to Exp. 2 and 3.
B	,,	29	,,	4	1			No	Control to Exp. 2 and 3.
C			,,	10	1			No	Biopsy near 6 A.
D			Spleen	11	2			No	Tip of spleen.
7 A	Intrav.	180				5	20	No	Control to Exp. 1 and 4.

* Each experiment represents one monkey.
† All monkeys were sacrificed and tissue collected.
‡ All parasites are in liver unless otherwise noted.

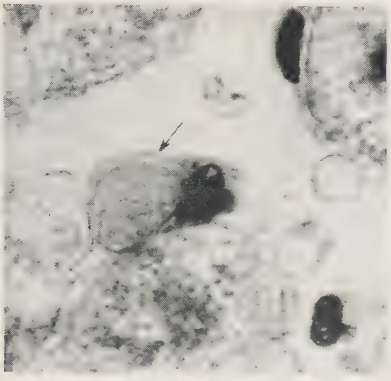

FIG. 1.
A pre-erythrocytic parasite of *P. cynomolgi* in a Kupffer cell, 6 days and 20 hours following the intravenous inoculation of sporozoites. Liver fixed in Zenker-formalin and stained by Maximow's hematoxylin, eosin-azur method. ×1300.

were found in cells which could not be identified; these may have been hepatic cells.

A splenic biopsy was also performed on this animal, 11 days and 18 hours after inoculation. Large bodies resembling schizonts and segmenters were observed in the venous sinuses, the pulp veins, and the trabecular veins. Despite this resemblance, the similarity of these forms to white thrombi raises a serious question as to whether they were exoerythrocytic stages. Heterophils, eosinophils, small and large lymphocytes, and monocytes were often observed surrounding and lying within these forms, resembling the leucocytic reaction which earlier investigators[7,10] had associated with segmenting exoerythrocytic stages of *P. cynomolgi*.

In *Exp. 3,* the liver of a monkey was inoculated in 2 places—in one site with 28 and in the other site with 27 heavily infected salivary glands. Liver biopsies were performed at 94 hours and at 144 hours but the inoculation site was located microscopically only in the 94-hour specimen. Schizonts and an occasional pre-segmenter were found in the 94-hour biopsy and ranged in length from 15-38 μ. These pre-erythrocytic parasites were primarily in Kupffer cells; some were definitely in hepatic cells. Three parasites were observed in the inoculation area in cells that could only be classified as large mononuclear phagocytic cells, similar to macrophages and Kupffer cells. The reaction area at the inoculation site was composed chiefly of degenerating liver cells, mosquito debris, heterophils, eosinophils, and a large variety of mononuclear phagocytic cells typical of an active inflammatory host response.

In *Exp. 4,* a monkey was inoculated intravenously with the ground thoraces of 223 heavily infected mosquitoes and was sacrificed 5 days and 19 hours later. Liver and spleen biopsies were made at 44 and 87 hours after inoculation. Air dried, Giemsa stained smears of the liver made at necropsy revealed the presence of 5 exoerythrocytic parasites, the largest of which had 26 chromatin bodies, the smallest 5. As is usual with such preparations, it was not possible to determine the type of host cell in which these parasites were located.

The parasites in these air dried liver smears contained vacuoles similar to those described for *P. cynomolgi*[7] and *P. relictum matutinum*.[13] However, no large vacuoles were seen in the histological preparations fixed in Zenker-formalin in any of the above experiments; clefts were observed.

In the biopsy made at 44 hours, small parasites resembling the small metacryptozoites of avian malaria were found in the Kupffer cells. This observation together with the findings of Experiment 2 suggest that cryptozoic segmentation occurred at about the 40th to the 48th hour.

A series of *control experiments* was performed to determine whether the observations mentioned above could be at all related to the parenteral inoculation of normal mosquito tissue. In this work, monkeys were inoculated, biopsied, and necropsied like the animals which received infected material. Tissues obtained from these animals were treated in the same manner as those from the infected monkeys. *Experiment 5* served as a normal control for the biopsies of infected liver and spleen. In *Exp. 6,* normal salivary glands were inoculated directly into the liver and then biopsied; this controlled Experiments 2 and 3. The monkey in *Exp. 7* controlled the infections, produced intravenously, in the

[13] Manwell, R. D., *Am. J. Trop. Med.*, 1940, **20**, 859.

animals of Exp. 1 and 4.

In none of these controls were bodies found which were similar to the pre-erythrocytic stages observed in the infected animals. The control material did demonstrate the need for caution in interpreting the data, for in these animals there were some forms such as degenerating liver cells and thrombi which might have been mistaken for parasites.

Discussion. The observations reported above suggest that the morphological characteristics of the pre-erythrocytic stages of *P. cynomolgi* are remarkably like those of the pre-erythrocytic stages of avian malaria. Certainly the tissue stages of *P. cynomolgi* are more similar to those of *P. gallinaceum* than of *Hepatocystes* (*Plasmodium*) *kochi*.[14] The exoerythrocytic stages of *Hepatocystes* resemble those stages observed in *Leucocytozoan* infections of ducks[15] while the *P. gallinaceum* and *P. cynomolgi* stages appear much like those of *Hemoproteus*. An obvious difference exists in that hepatic cells are not invaded by avian malaria and *Hemoproteus* whereas they may be in simian malaria, *Hepatocystes* and *Leucocytozoan*. Since *P. mexicanum* in the lizard[16] has both elongatum and gallinaceum type exoerythrocytic stages, it is to be expected that other species of malaria could have diverse host cell developmental potentialities.

Summary. Pre-erythrocytic stages of *P. cynomolgi* have been observed in Kupffer cells, hepatic cells, and mononuclear phagocytic cells of the liver, as well as in large mononuclear phagocytic cells of the spleen, probably reticular in nature.

[14] Garnham, P. C. C., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1948, **41**, 601.

[15] Huff, C. G., *J. Infect. Dis.*, 1942, **71**, 18.

[16] Thompson, P. E., and Huff, C. G., *J. Infect. Dis.*, 1944, **74**, 48.

16928

Determination of Carbon 14 in Fatty Acids by Direct Mount Technic.*

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The measurement of C^{14} in fatty acid fractions prepared from animals into which a C^{14}-labeled fatty acid has been introduced is beset with considerable difficulty. A sample of high specific activity is not often obtained because the administered radioactive fats are diluted by a factor of at least 1000 when mixed with the body pool of fatty acids. The activity per unit of mass is further reduced by a factor of 17 when the conventional $BaCO_3$ technics are used. The first dilution—that due to mixing with the body pool of fatty acids—obviously cannot be avoided. In order, however, to circumvent a further reduction of the activity, it seemed desirable to investigate direct mounting of fat samples.

When samples of C^{14}-containing fatty acids were mounted directly on bare aluminum discs, reproducible values for counting rates were not obtained because the material collected on the surface of the discs in the form of globules. Reproducible results were obtained by employing a cover of lens paper which brought about an even distribution of the fatty acids on the disc. The degree of reproducibility that can be attained by mounting radioactive fatty acids on lens paper-covered aluminum discs is shown in Table I which records the results of quadruplicate determinations for each of 3 different fatty acid samples. The deviations from the averages did not exceed 5%. The degree of reproducibility which can be at-

* Aided by a grant from the American Cancer Society (recommended by the Committee on Growth).

TABLE I.
Reproducibility of C^{14} Counting When Fat Was Mounted on Lens Paper-covered Aluminum Discs.
(In each experiment 4 one-cc aliquots of a solution of C^{14}-labeled palmitic acid in corn oil were separately mounted.)

Fatty acid sample	Counts per minute					% deviation from avg activity
	Per mount				Avg	
1	2780	2790	2780	2750	2775	0.5
2	3720	3610	3610	3810	3690	2.1
3	4430	4500	4380	4460	4440	0.8

tained in the counting of C^{14} by this method, when employed by different individuals was also investigated and it was found that the variation in the counts obtained by 3 individuals did not exceed 5%.

Although the method described above was designed primarily to avoid the dilution of activity inherent in the preparation of $BaCO_3$ mounts, its simplicity also permits a considerable saving of time. Fifty or more samples can be readily mounted by a single individual in a period of 8 hours.

A comparison of C^{14} activity as measured by direct mount technic with that measured as $BaCO_3$. It is frequently necessary to compare the specific activity of a fatty acid fraction with that of a non-lipid fraction or that of expired CO_2. Since at present the activity in the two latter materials is usually determined after conversion to $BaCO_3$, it became necessary to relate the activity of fat as measured by the direct mounting technic described below to its activity when measured in the form of $BaCO_3$.

Corn oil fatty acids were mixed with a sample of C^{14}-labeled palmitic acid and the mixture dissolved in petroleum ether. Suitable dilutions were then prepared so that each one-cc aliquot contained from 2.5 to 35 mg of fatty acids. Each sample was directly mounted and its activity determined. Duplicate samples were oxidized to CO_2 and converted to $BaCO_3$ and the activity of the $BaCO_3$ determined. All $BaCO_3$ counts were corrected to a standard mass of 40 mg according to the method of Henriques *et al.*[1]

[1] Henriques, F. C., Jr., Kistiakowsky, G. B., Margnetti, C., and Schneider, W. G., *Ind. Eng. Chem. Anal. Ed.*, 1946, **18**, 349.

The values for the ratio:

$$\frac{\text{Corrected counts from } BaCO_3}{\text{Counts from directly mounted fatty acids}}$$

were determined for many samples. A plot of these values as ordinates against mg of fatty acids used in the direct mount as abscissa is shown in Fig. 1. From this empirical curve, the factor for converting counts obtained from a direct mount to the activity that would have been obtained had the C^{14} of the fatty acids been measured after conversion to $BaCO_3$ can be read.

In order to test the reliability of the conversion factors, 5 samples of tissue fatty acids isolated from rats that had received C^{14}-labeled palmitic acid were mounted directly in duplicate and the activities determined. The values obtained were then converted to the $BaCO_3$ basis by using the factors in Fig. 1. Aliquots of these same 5 samples were then oxidized to CO_2 and their activities determined in the form of $BaCO_3$. The results are shown in Table II.

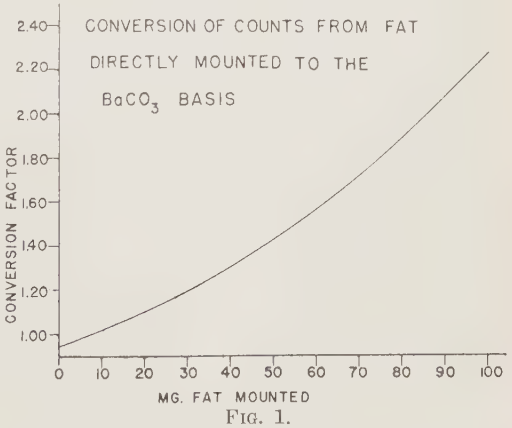

Fig. 1.

Factors for conversion of activity as measured by direct mounting technic to activity on a $BaCO_3$ basis.

TABLE II.
Reliability of Empirical Conversion from Counts per Minute per Mg Tissue Fatty Acids to Counts per Minute per Mg $BaCO_3$.

1	2	3
	Counts per Minute per Mg $BaCO_3$.	
Mg fatty acids	Calc. from direct mount by use of factors in Fig. 1	Found by oxidation of fatty acids to $BaCO_3$
5.7	10.8	10.3
5.7	10.6	10.2
12.9	10.8	10.3
13.3	10.4	10.1
17.9	11.0	11.1
18.1	10.6	10.3
22.5	10.9	10.3
22.9	11.0	10.3
28.1	10.9	10.5
27.9	11.1	10.1

The values obtained by this conversion (col. 2) are in good agreement with those obtained by direct oxidation of the fatty acids to $BaCO_3$ (col. 3). This agreement, therefore, justifies the use of the conversion factors and the determination of the radioactivity in fatty acids by the direct mount technic, a procedure that yields the highest activity per unit of mass.

Experimental. Direct mounting of fatty acids on lens paper-covered aluminum discs. An aluminum disc 1.75 inches in diameter, lined with a piece of lens paper of the same diameter, was weighed and then placed approximately 6 inches below an infra-red lamp. An aliquot of a fatty acid solution (usually one cc) containing the C^{14}-labeled fatty acid was added dropwise to the warmed lens paper at a rate that kept the surface constantly and uniformly wet but prevented the fat solution from creeping beyond the edge of the disc. The disc and its contents were then reweighed. It was found that, by this method, about 40 mg could be safely mounted on a single disc without encountering loss from creeping. If a concentrated solution of fatty acids in ether is evaporated by using an air stream instead of an infra-red lamp, however, as much as 150 mg of fatty acids can be mounted on a disc. The activity of the mounted material was measured by a thin mica window Geiger tube.

Satisfactory results with lens paper which spreads the solution evenly have been reported by Fager.[2] His procedure, however, differs from that described above.

Wet combustion of fatty acids. The apparatus (Fig. 2) used for wet oxidation is a modification of that described by Skipper *et al.*[3] Potassium iodate was omitted from the combustion fluid. Concentrated H_2SO_4 or the combustion fluid was used as a joint lubricant. The tapered joints of the standard glass-stoppered 125 cc Erlenmeyer flasks occupy a portion of the 19/38 joints (Fig. 2). The samples were pipetted into the flasks and the solvent evaporated on the steam bath, the last traces being blown out with nitrogen. After the apparatus was assembled, the vacuum line was opened and CO_2-free air drawn through at a rate sufficient to provide the necessary dispersion in the NaOH tower. A measured amount of the combustion fluid was then added through the funnel, and the flask heated gently until fumes first appeared. This temperature, or a slightly lower one, was maintained for 5 minutes. Care was taken to avoid the excessive production of SO_3 fumes.

[2] Fager, E. W., Reported in Symposium on the Use of Isotopes in Biological Research, University of Chicago, March, 1947.

[3] Skipper, H. E., Bryan, C. E., White, L., Jr., and Hutchinson, O. S., *J. Biol. Chem.*, 1948, **173**, 371.

The flame was removed and CO_2-free air passed through the apparatus for an additional 10 minutes. The $Na_2C^{14}O_3$-NaOH was next forced into a volumetric flask by means of air pressure. Aliquots were taken for the precipitation and mounting of $BaCO_3$ and for the determination of CO_2.

The completeness of oxidation under the conditions described above was tested for the following compounds: palmitic acid, cholesterol, glucose, and corn oil. Six mg of the first 3 compounds were used whereas corn oil was tested over a range of 5 to 30 mg. In all cases, the recovery of CO_2 was from 95 to 100% of the theoretical.

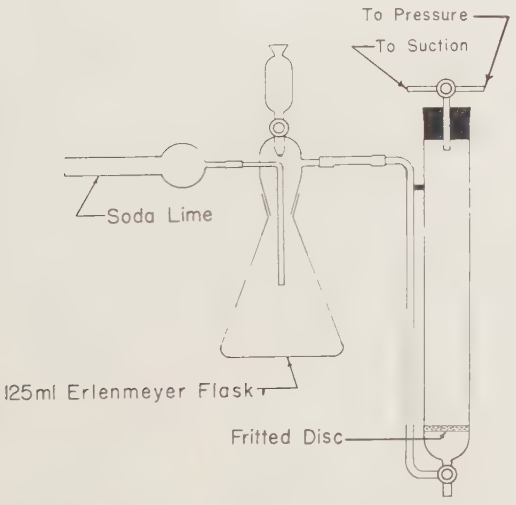

Fig. 2.
Apparatus used for oxidation of organic compounds.

Determination of CO_2. An excess of a saturated $BaCl_2$ solution was added to aliquots of the $Na_2C^{14}O_3$ solution. This solution was then titrated with dilute HCl to the phenolphthalein end point. Brom cresol green was then added, and the solution titrated with standard 0.1 N HCl to the new end point. The amount of $BaCO_3$ precipitated was calculated from the titration difference which had been corrected for a blank titration value.

Preparation of the $BaCO_3$ mount.† An excess of a saturated $BaCl_2$ solution was added to an aliquot of the $Na_2C^{14}O_3$ solution. Suction was applied to the filtration apparatus (Fig. 3) and the suspension of $BaCO_3$ poured into the glass cylinder. The suspension was allowed to filter completely and the precipitate, while still moist, was washed first with water and next with acetone. Suction was maintained constantly. After removal of the glass cylinder, an infra-red lamp was placed one inch above the surface of the mount and hot air was pulled through the mount for several minutes. Suction was discontinued at this point. The paper and $BaCO_3$ were removed as a flat mount. (There should be little or no curvature.) For determination of its radioactivity, the mount was held flat by means of the carrier shown in Fig. 4. No loss of activity has been observed in samples kept in covered lucite trays for as long as 11 weeks.

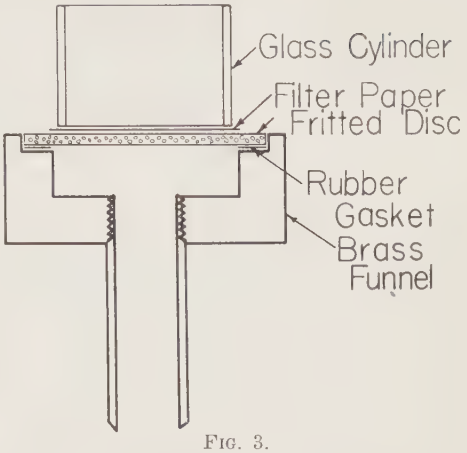

Fig. 3.
Filtration apparatus for preparation of $BaCO_3$ mounts. The filter paper used is Whatman No. 42. The glass cylinder is held firmly in place by means of two elastic bands attached to glass ears on the sides of the cylinder and to metal screws on the sides of the brass funnel.

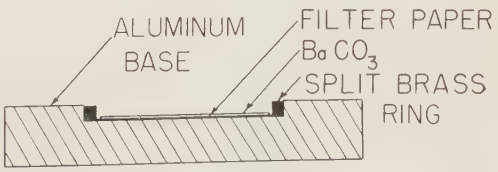

Fig. 4.
Holder for keeping $BaCO_3$ filter paper mounts flat during counting.

† Similar mounting technics have been described for benzidine sulfate[1] and P^{32} in plant material.[4]

[4] MacKenzie, A. J., and Dean, L. A., *Anal. Chem.*, 1948, **20**, 559.

TABLE III.
Reproducibility of C^{14} Determination When $BaC^{14}O_3$ Was Mounted on Filter Paper.

Sample	$BaCO_3$ mounted, mg	Counts per min.		Specific activity of $BaCO_3$, counts per min. per mg	% difference between duplicate specific activities*
		Observed	Corrected for mass		
A	57.3	725	860	15.0	4.5
A	57.8	765	910	15.7	
B	76.1	1370	1920	25.2	1.6
B	74.5	1330	1850	24.8	
C	54.5	925	1060	19.5	4.5
C	53.4	955	1090	20.4	
D	43.5	329	342	7.9	3.9
D	44.1	323	336	7.6	
E	43.4	143	149	3.4	2.9
E	43.0	148	152	3.5	

* These values include errors of titration and counting.

After its radioactivity had been determined, the $BaCO_3$ mount was transferred to an Erlenmeyer flask and an excess of standard 0.1 N HCl added. After the reaction was complete, the excess acid was titrated to the brom cresol green end point with standard alkali. The weight of the $BaCO_3$ was calculated from the equivalents of acid consumed.

For permanent mounts, or as an alternative to the $BaCO_3$ titration procedure, the filter paper can be weighed before and after the $BaCO_3$ is mounted. For samples of small weight, the filter paper should be dried at 120° before each weighing.

In order to test the reliability of the mounting of $BaCO_3$ on filter paper, the following experiments were carried out. Five $Na_2C^{14}O_3$ solutions (A to E, Table III) of varying radioactivity were used. From each solution, 2 mounts were prepared on filter paper (as described above) and their C^{14} activity determined. The weight of the $BaCO_3$ of each mount was calculated from titration values. The 2 values found for the specific activity of each sample (Table III) were in good agreement, no pair differing by more than 5%.

The data presented here apply only to the particular geometry of the counting device employed in this laboratory and hence cannot be applied to cases where the counting arrangements differ.

Summary. It is demonstrated in the present investigation that the C^{14} activity of a fatty acid sample can be readily determined by a direct procedure that avoids the dilution of activity and laboriousness associated with the preparation of $BaCO_3$ mounts. The simplicity of this new procedure permits a single operator to mount as many as 50 samples in 8 hours with an error of reproducibility not in excess of 5%. By means of an empirically constructed curve, the observed activities can be converted to a $BaCO_3$ basis.

16929

Leucocyte Blockade of *in vitro* Tuberculin Cytolysis.*

CUTTING B. FAVOUR. (Introduced by J. H. Mueller.)

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It has recently been shown that a portion of the white blood cells taken from a person acutely ill with tuberculosis are "lysed" by amounts of tuberculin not toxic for white blood cells taken from normal subjects or from individuals suffering from a variety of other acute illnesses.[1] It has also been found that this cytotoxic effect of tuberculin involves only the lymphocyte in the mouse,[2] a species which shows no intracutaneous sensitivity to tuberculin[3] whereas both lymphocytes and granulocytes are destroyed *in vitro* in the blood of man and guinea pigs showing *in vitro* cytolysis. A possible explanation for this discrepancy may be a difference in the affinity of human and mouse white blood cells for tuberculin. The experiments to be described are presented to show that indeed human white blood cells do and mouse white blood cells do not adsorb tuberculin.

Materials. 1. Mouse tuberculosis. Six-week-old albino Swiss mice were inoculated intravenously with 0.1 cc of a 6 to 10 day culture of H37Rv grown in synthetic media.[4] In this experiment 60% of the animals died within 6 weeks. Those surviving at 2 to 4 months were individually exsanguinated from the heart and the spleens from selected mice with gross evidence of advanced pulmonary tuberculosis removed and minced in an 0.85% saline solution containing 200 mg% glucose. Cell suspensions free of tissue fragments were washed three time by centrifugation and resuspension in the same solution and the cell count adjusted to 200,000 cells per cu. mm.

2. *Human tuberculosis patients* hospitalized for acute tuberculous infections were bled without stasis from the antecubital vein into a syringe containing 1 ml (10 mg) of heparin solution (Roche Organon). The 20 ml of blood was allowed to stand at room temperature in a test tube placed at an angle of 20-30°. Within 20 minutes the sedimentation was sufficient to give an almost quantitative separation of red cells from the plasma. The plasma, containing the majority of the white blood cells, was removed and centrifuged lightly to sediment the leucocytes. The supernatant containing most of the platelets was removed, centrifuged for 20 minutes at 2000 RPM to remove the platelets and its supernatant used to resuspend the homologous white blood cells. The cell concentration was adjusted to 200,000 cells per cu. mm.

3. *Normal mouse* white cells were obtained in the same fashion as tuberculous mouse white cells. Normal human white cells were obtained in a similar manner to that used for cells from tuberculous patients. A rapid sedimentation rate was induced in the normal human blood after heparinizing by adding 2 ml of a physiological solution of fibrinogen solution† per 20 ml of blood.

4. *Tuberculin.* O. T. obtained from the Massachusetts Department of Health was dialyzed against three changes of 0.85% saline during 3 days and the volume adjusted so that the concentration used represented a 6-fold dilution of the original material.

Heparinized blood from normal and tuberculous mice and humans was obtained by cardiac and venipuncture respectively immediately prior to setting up the experiment.

Method. A sample protocol illustrates the

* Work done under a U.S.P.H.S. Research Grant.

1 Fremont-Smith, P., and Favour, C. B., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 502.

2 Favour, C. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 269.

3 Gerstl, B., and Thomas, R. M., *Yale J. Biol. and Med.*, 1940-41, **13**, 679, ref. cit.

4 Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

† Fifteen mg of the lyophilized Fraction I of Cohn per ml of distilled water.

	Normal			Tuberculous		
	T_1	S_1	P_1	T_2	S_2	P_2*
Normal cells	.8 ml	.8				
" plasma			.5			
Tuberculous cells				.8	.5	
" plasma						.5
O.T.	.2		.2	.2		.2
Saline		.2			.2	

Incubated at 37°C for 30 min.
T_1, S_1, T_2, S_2 centrifuged 10 min. at 2000 RPM.
The supernatants removed and used in procedure below.

	I	II	III	IV	V	VI	VII	VIII	IX
Man:									Normal blood
Heparinized tuberculous blood	.4 ml	.4	.4	.4	.4	.4	.4	.4	.4
Supernatant T_1	.1								
" S_1		.1							
" P_1			.1						
" T_2				.1					
" S_2					.1				
" P_2						.1			
O.T. 1:2							.1		.1
Saline								.1	
WBC 5′	4530	4020	4440	4670	4520	4290	4000	4020	3140.
WBC 60′	4400	4000	2880	4550	4340	3430	2610	3940	3010
% difference	−3.0	0	−35.1	−2.5	−3.8	−20.7	−34.7	−2.1	−2.1
Mouse:									Normal blood
Spleen cells	5020	4280		5100	4390		5100	4770	2700
	3390	4340		3450	4420		3360	4770	2700
	−33.5	+1.2		−32.3	−0.8		−34.0	0	0

* T = tuberculin + cells.
S = saline + cells.
P = tuberculin + plasma.
1 = normal
2 = tuberculous.

method of experimentation. The tubes and counting procedures used were the same as described in a recent communication.[5] In the experiments using human blood, subjects with the same blood groups were used.

The illustrated experiment has been carried out 2 or more times on 4 different subjects and 3 separate pools of mouse spleen cells with essentially identical findings. In many other experiments done to explore the optimal concentrations of various ingredients, it has been found that the cell concentrations, amounts of O.T. used, and the duration of incubation are critical. Although complex, the experiment can be repeated regularly when the indicated precautions are observed.

"Full strength" O.T. is used in the first section to equal in final dilution that obtained with O.T. 1:2 in the second section.

Results. Inspection of the table indicates that the plasma suspension of white blood cells of normal and of tuberculous people blocks tuberculin when an *in vitro* biological assay method for the detection of tuberculin is used. White cells from the spleen of a tuberculous mouse do not so block tuberculin. In neither species does the presence or absence of tuberculosis in the donor host alter the type of *in vitro* cell-tuberculin-blockade. It is interesting, however, that the serum of a normal

[5] Favour, C. B., and Fremont-Smith, P., to be published.

person does not inactivate tuberculin whereas the tuberculous individual's blood does partially block the subsequent *in vitro* cytotoxic effect of tuberculin.

Comment. It has long been assumed that the tuberculin which is placed in the skin or other tissues and which produces a delayed tuberculin reaction is localized in that area. The very nature of the tuberculin reaction itself is evidence for this belief. In the experiments reported here some direct evidence for this belief is presented. The leucocytes of man which are the classic model of host cells of the tissue culture worker are capable of reacting with tuberculin in such a way that it no longer has cytotoxic effects on sensitized white cells from tuberculous subjects. Mouse leucocytes, on the other hand, do not react with tuberculin. It is perhaps not a coincidence that the tuberculous mouse shows no intracutaneous reactions to tuberculin[3] as do tuberculous rabbits, guinea pigs and humans. These findings suggest that there is a primary "affinity" for tuberculin of the tissues of some species and not for others. This "affinity" does not depend on the presence of a tuberculous infection but is present in those species capable of developing a tuberculin reaction. This phenomenon may be compared to other tissue tropisms, for example the neurotropic viruses. It also helps to explain some of the differences in cell damage produced in the tuberculin type reaction as contrasted to the cellular changes in anaphylactic phenomenon.

Summary. 1. Suspensions of human white blood cells in homologous fresh plasma will adsorb tuberculin (O.T.) from the plasma.

2. White blood cells from normal persons with a negative intracutaneous reaction to tuberculin as well as white cells from persons with active tuberculosis show this phenomenon.

3. Suspensions of mouse white blood cells in homologous fresh plasma will not adsorb tuberculin from the plasma.

4. There is no difference in the lack of tuberculin adsorption by white blood cells from normal or tuberculous mice.

5. Normal human plasma does not whereas plasma from patients with active tuberculosis does partially inactivate tuberculin under the conditions of these experiments.

6. Tuberculin has been assayed by its *in vitro* cytotoxic effect on white blood cells from tuberculous subjects.

16930

Failure of Phenosulfazole to Influence the Course of Infection with Murine Poliomyelitis Virus in Mice.

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Sanders, SubbaRow, and Alexander[1] presented evidence to show that a new sulfonamide compound (N-(2-thiazolyl)-phenol sulfonamide, abbreviated as phenosulfazole, trade name "Darvisul") which was synthesized at the Calco Chemical Division of the American Cyanamid Company acted as an effective antiviral substance in mice infected with the Col. SK strain of murine poliomyelitis virus. Drug treatment instituted 24 hours following intraperitoneal injection with virus at various levels of potency apparently brought about a regular and striking reduction in the mortality rate when compared with that among untreated controls. As judged from the protocols, however, the therapeutic effect showed no clear gradations with respect to amount of virus used for infection or

1 Sanders, M., SubbaRow, Y., and Alexander, R. C., *Texas Rep. on Biol. and Med.*, 1948, **6**, 385.

TABLE I.
Attempted Chemotherapy of Murine Poliomyelitic Infection with Phenosulfazole.

Exper.	Seed virus	Mice	Intraperitoneal infection* 10-1	10-2	10-3	10-4	10-5	10-6	10-7	10-8
I	Mouse passage	Drug treated (8-10 g)					10/10	10/10	10/10	8/10
	Stock virus (glycerinated)	Controls ,, ,,					10/10	10/10	10/10	8/10
II	Mouse passage	Drug treated (18-20 g)				10/10	10/10	10/10	6/10	
	Stock virus (fresh)	Controls ,, ,,				10/10	10/10	8/10	5/10	
III	Guinea pig	Drug treated (14-18 g)	10/10	9/10	10/10	0/10				
	Virus (glycerinated)	Controls ,, ,,	10/10	10/10	8/10	0/10				
IV	First return mouse	Drug treated ,, ,,				9/10	10/10	9/10	9/10	
	Passage from guinea pig (glyc.)	Controls ,, ,,				10/10	10/10	10/10	7/10	
V	Tissue culture	Drug treated ,, ,,	9/10	4/10	0/10					
	Virus (8th serial passage) (fresh)	Controls ,, ,,	8/10	1/10	0/10					
VI	First return mouse	Drug treated ,, ,,				10/10	10/10	10/10	10/10	
	Passage from tissue culture (fresh)	Controls ,, ,,				10/10	10/10	10/10	10/10	
VII	First return mouse	Drug treated ,, ,,				10/10	10/10	10/10	10/10	
	Passage from tissue culture (frozen)	Controls ,, ,,				10/10	10/10	10/10	10/10	

Note: Results are recorded as follows: Numerator = No. of mice paralyzed or dead. Denominator = No. of mice injected. The incubation periods were not significantly different between drug-treated and control animals. They varied in the different experiments between 3-10 days depending on the type and dilution of virus used.

* The infectious dose was 0.1 cc of the indicated virus dilutions in Experiment I. In all subsequent experiments (II-VII) the dose was 0.06 cc.

dosage of drug employed for treatment. Thus, the percentage of drug-treated surviving animals hovered around 50% irrespective of whether the mice had previously been infected with approximately 1000 ID_{50} (10^{-4}) or 1 ID_{50} (10^{-7}). Again, there was no uniform improvement of the survival rate when the total amount of drug, administered in fractional daily doses over a period of 5 consecutive days, was stepped up from 40 mg to 80 mg. The following report deals with an unsuccessful attempt to verify the above results.

The drug (lot 7-8522 and lot 7-8564) was obtained through the courtesy of Lederle Laboratories, Inc. The drug was administered, 16 to 18 hours after intraperitoneal infection, by intraperitoneal injection of 4 daily divided doses as follows: 5 mg, 4 mg, 4 mg, 5 mg. The total amount of drug per diem was therefore 18 mg. This treatment was continued for 4 to 5 days. No symptoms of toxicity were observed with this dosage. Controls received 0.85% NaCl solution instead of drug. The mice were Rockland Swiss albino mice weighing, with one exception, from 14 to 20 g. The age of the animal is important because older mice are usually less susceptible by peripheral inoculation. Col SK murine virus was used in 3 forms: 1) mouse brain passage virus,)2 guinea pig brain virus and virus harvested from first return mouse passage, 3) tissue culture virus and virus harvested from first return mouse passage. One reason for choosing as infecting agent more than one source of virus was the fact that the 3 different strains—and their subpassages—represent stabile and unstabile forms of the same virus, with varying degrees of peripheral invasiveness,[2-4] which might conceivably respond selectively to the drug. Another more practical consideration was our desire to work under conditions identical with those employed by Sanders, SubbaRow, and Alexander, who operated with a seed virus obtained by passage of tissue culture virus to mice. In all other important details we followed closely the procedure described by these investigators. A total of 7 different experiments were run which are summarized in Table I.

From the data given in Table I it appears that phenosulfazole in adequate dosage had no demonstrable chemotherapeutic effect on the course of infection in mice induced with graded doses of Col SK virus under a wide variety of experimental conditions. It will further be noted that the drug not only failed to manifest any direct antiviral effects against the stabile forms of the virus (mouse passage virus, guinea pig virus, tissue culture virus), but likewise failed to display any indirect effects on the unstabile forms of the virus (first return mouse passage from infected guinea pig or tissue culture medium). In other words, there was nothing in our work to indicate that the drug has the power to cause reversion of the high peripheral infectivity of such viral forms to the low level of peripheral infectivity of their parent viral forms, a phenomenon which, if true, might have furnished a possible explanation for the curious observations reported by Sanders, SubbaRow, and Alexander. We are, therefore, at a loss to reconcile our own negative findings with the positive results reported by the earlier investigators.

Conclusions. Phenosulfazole had no demonstrable effect on the course of infection with murine SK poliomyelitis virus in mice.

2 Sanders, M., and Jungeblut, C. W., *J. Exp. Med.*, 1942, **75**, 631.

3 Jungeblut, C. W., Feiner, R. R., and Sanders, M., *J. Exp. Med.*, 1942, **76**, 31.

4 Schultz, E. W., and White, S. C., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 266.

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